Multiplex detection for sexually transmitted infections using a novel electrochemical assay

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Chapter 1 - Abstract

Chlamydia trachomatis (Chlamydia) is the most common sexually transmitted infection diagnosed in genitourinary medicine (GUM) clinics and affects one in ten sexually active young people. *Neisseria gonorrhoeae* (gonorrhoea) is the second most common. They are asymptomatic in at least three-quarters of patients. Untreated infection can lead to serious health problems; including pelvic inflammatory disease and infertility in women. In men, it can cause urethritis and Reiter's Syndrome (arthritis). The number of diagnoses of uncomplicated chlamydia in GUM clinics in England increased by 288% between 1995 and 2006[1].

Co infection with chlamydia and gonorrhoea is not uncommon. Up to half of patients diagnosed may be infected with both pathogens and, therefore, it is important to test sexually active individuals for both chlamydia and gonorrhoea using a multiplex system[2].

Electrochemical detection of specific DNA sequences offers advantages over established fluorescence techniques; including cost and ease of miniaturisation. The electrochemical gene sensor described here uniquely utilises enzymatic T7 Exonuclease digestion to generate ferrocenylated oligonucleotide fragments in a matched (gene sequence presence) sensor. These fragments exhibit a characteristically high response upon electrochemical analysis, using Differential Pulse Voltammetry and have been designed based on the ability to 'tune' the oxidation potential of ferrocene by using electron-withdrawing and electron-donating substituent groups. Ferrocenylated oligonucleotide labels have been synthesised, each with a characteristic oxidation potential.

Results from a study of the adsorption and electron transfer kinetics of T7 exonuclease - digested and undigested oligonucleotide redox probes at screen-printed carbon paste electrode (SCPE) surfaces will be considered as a basis for the assay discrimination. The high selectivity of the T7 Exonuclease digest is demonstrated using the ferrocenylated oligonucleotides and a triplex genomic electrochemical assay for the detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and the human

beta-actin gene as an internal control for the assay using clinical samples will be presented.

Chapter 2 - Introduction

2.1 The Biosensor

A Biosensor is defined as an analytical device incorporating a biological material, a biologically derived material or a biomimetic intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical[3].

Biosensors usually yield a digital electronic signal which is proportional to the concentration of a specific analyte or group of analytes and as such, biosensors have been applied to a wide variety of analytical problems including in medicine, drug discovery, the environment, food, process industries, security and defense[3].

Clinical Diagnostic biosensors are established entities within the healthcare sector as they are integral to the detection and management of disease. Until c.1980, bacterial culture was the only method for establishing diagnoses of certain infections and the technique is still in use in medico-legal cases today due to its high-specificity[4]. Bacterial culture, however, remains time-consuming and highly-specialised. In 1986, Enzyme immunoassay (EIA) was introduced as a detection method for Chlamydia[5]. The method proved less expensive and more user-friendly; however it did present specificity issues[5]. The objective of a large quantity of current research in the fields of virology, bacteriology and biosensor systems is to evaluate the clinical applicability of multiplex polymerase chain reaction (PCR), as detailed further in section 3.5.2. Brittain-Long *et al.* for example, have recently developed a real-time PCR procedure for the detection of respiratory tract infections, based on automated specimen extraction and multiplex amplification, at a relatively low cost (€33)[6]. This particular research also confirmed that it is often very difficult for the clinician to distinguish between viral and bacterial aetiologies, and this may result in the overuse of antibiotics[7]. Furthermore, the study detailed that diagnosis of viral respiratory tract infections using viral culture, antigen detection or serology is either too slow or too insensitive to be applicable in clinical practice[8]. Nucleic acid amplification-based methods are now of prime importance for the diagnosis of bacterial infections[9]. A

number of commercial products using nucleic acid amplification technology are now available[10]. These tests amplify the target nucleic acid, DNA or ribonucleic acid (RNA); or the probe after it has annealed to target nucleic acid. Such tests are generally more sensitive than liquid or solid phase hybridisation tests which do not embody an amplification process[10] and are considerably more sensitive than culture or antigen detection methods[11].

2.2 Sexually Transmitted Infections – *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG)

2.2.1 *Chlamydia trachomatis*

According to the United Kingdom (UK) Health Protection Agency (HPA), Genital chlamydial infection remained the most commonly diagnosed bacterial sexually tramsmitted infection (STI) in UK genitourinary medicine (GUM) clinics in 2006, comprising 30% (113585 / 376508) of all new STI diagnoses in this setting. If untreated, Genital chlamydia can lead to pelvic inflammatory disease, ectopic pregnancy and tubal infertility in women and can affect sperm function and fertility in men. At least 70% of women and 50% of men with chlamydial infection show no symptoms and may remain undiagnosed in the absence of screening for asymptomatic infection[12]. The annual cost of chlamydia and its consequences in the UK is estimated to be more than £100 million[13].

Countries within the UK vary in their approaches to prevention and control of chlamydia. In Scotland there is no specific centrally led programme but clear guidance is given to health professionals in the form of the evidence-based Scottish Intercollegiate Guidelines Network[14]. These will be updated in 2008 but currently recommend the testing of all patients attending GUM clinics, all women undergoing termination of pregnancy, all symptomatic men and women in any clinical setting, and opportunistic screening of all sexually active women aged under 25 and women aged over 25 with two or more partners or a change of partners in the previous year[15].

Since 2003, there has been a proactive policy in England, the National Chlamydia Screening Programme (NCSP), which is a key part of the government's strategy for the modernisation of sexual health services. Its introduction has occurred in three phases to enable managed growth. Full roll out to the 152 Primary care Trusts (PCTs) in England will be almost completed by the end of 2007. The NCSP targets all sexually active men and women under 25 with multifaceted opportunistic screening involving a diverse range of settings in both healthcare and non-healthcare locations. Effective partner notification to reduce the risk of re-infection and reinforce health education messages is integral to the programme[15].

2.2.2 *Neisseria gonorrhoeae*

Gonorrhoea is the second most common bacterial sexually transmitted infection in the UK which, if untreated, can lead to complications such as chronic pelvic pain, ectopic pregnancy and infertility in women. In 2006, there were 19007 diagnoses of uncomplicated gonorrhoea in GUM clinics in the UK, 13627 in men and 5380 in women. This translates to an overall rate of 46 and 18 per 100000 population in men and women respectively[16]. Numbers and rates of gonorrhoea diagnoses are highest in men partly because men are more likely to be symptomatic, but also because a significant proportion of cases are acquired through sex between men[17].

Targeted interventions are particularly appropriate for gonorrhoea as it is concentrated in specific population sub-groups[18]. As the great majority of gonorrhoea diagnoses are still made in GUM clinics, reinforcing and enhancing primary and secondary prevention activities within this setting could have a significant impact on infection control. Indeed, recent improvements in patient waiting times[19], the increase in numbers of sexual health screens being done, and evidence of improved partner notification within the GUM clinic setting may all have helped interrupt heterosexual transmission of gonorrhoea in recent years[20, 21].

Outside of the GUM clinic setting, there is increasing evidence that in certain localised areas where chlamydia screening is being introduced, some sites are also testing specimens for gonorrhoea[22, 23, 24]. The evidence base for wider use of

gonococcus nucleic acid amplification tests is growing and it is proposed that their use should be the subject of formally designed studies so as to better inform the development of national policy[25].

2.3 Emerging Clinical Requirements for Point of Care Diagnostic Testing for CT and NG.

Clinical Biochemistry produces and interprets the results of chemical and biochemical analyses performed on blood and other body fluids to help in the diagnosis and management if disease[26]. Consequently, sensors for bacterial pathogens have advanced rapidly within this sector of public healthcare. A survey of General Practitioners in England and Wales by the Public Health Laboratory Service indicated that, among infectious diseases, the greatest opportunity for improving public health was seen to be the better management of chlamydial genital tract[27]. Improved availability of diagnostic tests for chlamydial infections was also a major priority[28]

Co-infection with CT and NG is not uncommon. In fact, up to half of patients diagnosed may be infected with both pathogens and, therefore, it is important to test all sexually active individuals for both CT and NG[29].

Figure 1 illustrates the current rates of increase in newly diagnosed cases of CT and NG.

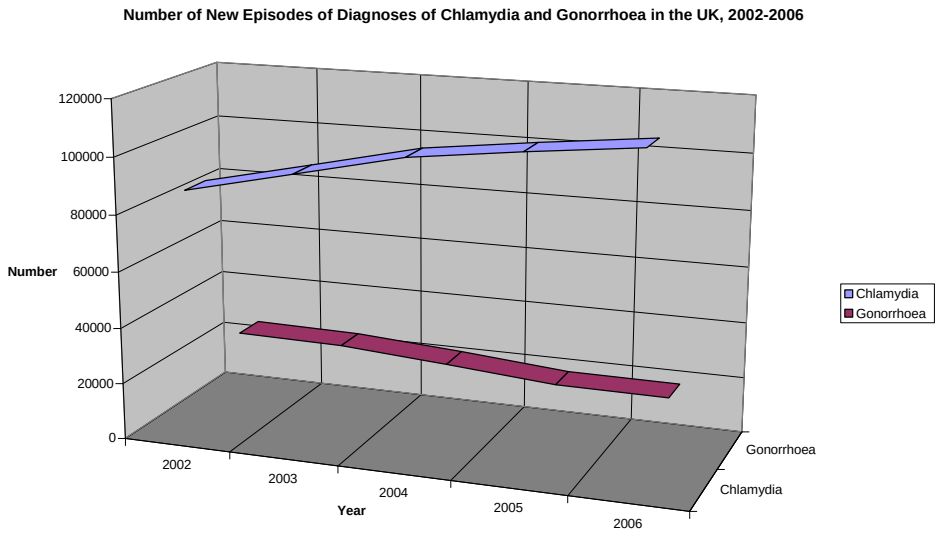


Figure 1: The Number of New Episodes of Diagnoses of Chlamydia and Gonorrhoea in the UK, 2002-2006. Source: UK Health Protection Agency Annual 2006 STI Data.

The high prevalence of asymptomatic gonoccocal and chlamydial infections is one of the greatest obstacles to STI control, especially in developing countries, where partner notification is difficult. A widely available diagnostic test which allowed prompt and effective treatment of asymptomatic patients could reduce the prevalence of these infections, prevent complications, and reduce the incidence of HIV infection, whose transmission they facilitate. Such a test could also play an important part in reducing unnecessary treatment of patients with STI syndromes that are not caused by these pathogens[30].

Most current tests for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* require the support of a laboratory, and results are not usually available before the patient has left the clinic. This delay can lead to patients not returning for treatment and may allow further STI transmission to occur. Current rapid point of care (POC) STI tests and the syndromic approach are less sensitive than gold standard tests, but allow treatment at the initial visit.

Vickerman *et al*[31] developed a mathematical model to compare the STIs averted using different STI diagnostic metods. The results of this study indicated that the required sensitivity of a POC test is low if there is significant STI transmission during the delay in treatment for the gold standard test and/or few patients return for treatment.

For example, the required sensitivity of a POC test for *C. trachomatis* is 50% (gold standard sensitivity is 90%) if either 55% return for treatment and there is no STI transmission, or 80% return for treatment and 50% of infected patients infect their partner during the delay in treatment. Furthermore, in these settings a POC test of moderate sensitivity can lead to significantly more STI averted than the gold standard test.

These results support the use of moderate sensitivity POC tests in scenarios where many patients will not return for treatment, and in populations where the delay in treatment would result in significant STI transmission[32].

2.4 Established Methodology for STI Diagnosis

2.4.1 Cell Culture Assays

Until the early 1980s, the main method of confirming a diagnosis of chlamydial infection was the inoculation of clinical material into animals, or more commonly, tissue culture cells and the demonstration of characteristic chlamydial inclusions. Usually, inclusions were demonstrated in cell culture, either by staining with iodine, Giemsa stain, fluorochrome-labelled poly- or mono-clonal antibody or by enzyme immunohistochemistry. The procedures involved in diagnosis by cell culture meant that chlamydial diagnoses were performed by a few specialist research laboratories. The main advantage was that identification of even one chlamydial inclusion was usually sufficient to establish a definitive diagnosis. Even now cell culture is one reference standard against which new diagnostic tests can be compared and it is still the method of choice for medico-legal cases, for example child sexual abuse. Tissue culture services are however labour intensive and expensive to maintain with many variables including the tissue culture water, the particular clone of cells in use and the elimination of adventitious mycoplasma infection[33].

2.4.2 Immunoassays

Demanding, but less variable protocols associated with modern, non viability-dependent diagnostic products with better sensitivity than was routinely achieved through cell culture techniques have since been developed[33]. The first commercial enzyme immunoassay (EIA) for the detection of chlamydial antigen in clinical specimens was the Abbott Chlamydiazyme®[34]. The Chlamydiazyme was based on an adsorbed polyclonal antiserum, rather than a monoclonal antibody. It soon became clear that there were specificity problems, however, associated with the finding that the enzyme immunoassay antibody reacted with strains of *Acinetobacter calcoaceticus*, *Escherichia coli*, *Gardnerella vaginalis*, *Neisseria gonnorrhoeae* and group B streptococci[35]. In women, urinary tract infection could cause false positive results[36]. However, specificity was improved by the introduction of a confirmation test blocking antibody[37].

The Boots-Celltech (subsequently DAKO) IDEIA® was introduced to the market as a radically different immunoassay, being based on the improved specificity of monoclonal antibody to the chlamydia-specific lipopolysaccharide epitope plus a novel redox-linked, signal amplification system[38]. Early results suggested that the sensitivity and specificity of these assays for use on endocervical or endourethral specimens were broadly comparable[39].

A range of rapid, qualitative enzyme immunoassay-based diagnostic tests for chlamydial antigen were also developed, aimed at point of care diagnosis; including the Unipath Clearview®, the Kodak Surecell® and the Quidel Quickview®. The general impression is that these qualitative tests had lower sensitivity than their less rapid but quantitative counterparts. Rani *et al*[40] conducted a pilot study to evaluate the sensitivity and specificity of the Quidel Quickview® Chlamydia test in populations with a low and high prevalence of chlamydial genital tract infection. Furthermore, these results were compared to PCR for endocervical samples. The sensitivity and specificity of the QuickVue test compared to PCR was 65% and 100% respectively for the high prevalence population and 25% and 100% respectively for the low prevalence population. The sensitivity of the QuickVue test for a high prevalence population was comparable to laboratory-based enzyme immunoassay techniques, but

not to nucleic acid amplification based methods. However, for a low prevalence population, this test, due to low sensitivity, failed to identify one in four cases.

Many laboratories still use chlamydial antigen detection immunoassays because of their ease of use and lower cost. However, research interest in this methodology waned with the introduction of superior methods based on nucleic acid hybridisation and amplification.

2.5 DNA Methods

2.5.1 Deoxyribonucleic acid (DNA)

Deoxyribonucleic acid (DNA) is a nucleic acid that contains the genetic instructions specifying the biological development of all cellular forms of life and many viruses. DNA is a polymer with monomer units called nucleotides. Hence, the polymer is known as a "polynucleotide." Each nucleotide consists of a 5-carbon sugar (deoxyribose), a nitrogen-containing base attached to the sugar, and a phosphate group. There are four different types of nucleotides found in DNA, differing only in the nitrogenous base, given one letter abbreviations as shorthand for the four bases. Adenine (A), Guanine (G), Cytosine (C) and Thymine (T). Adenine and guanine are substituted purines and cytosine and thymine are substituted pyrimidines. In 1953, James Watson and Francis Crick proposed the now classic secondary structure of DNA[41]. According to the Watson - Crick Model, DNA consists of two polynucleotide strands coiled around each other in an anti-parallel direction to form a double helix (Figure 2). The strands are connected by bases, hydrogen bonded together in a highly specific way. A and T form strong hydrogen bonds to each other but not to C or G, while C and G form strong hydrogen bonds to each other but not to A or T (Figure 3).

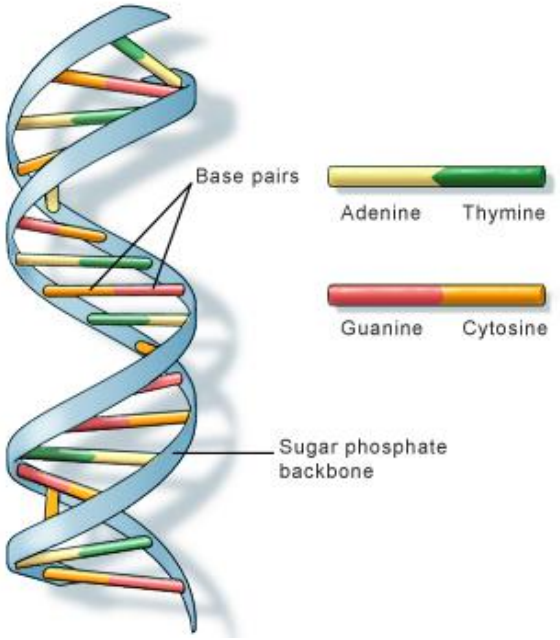


Figure 2: The Double Helix Model of DNA. Image courtesy of U.S. National Library of Medicine.

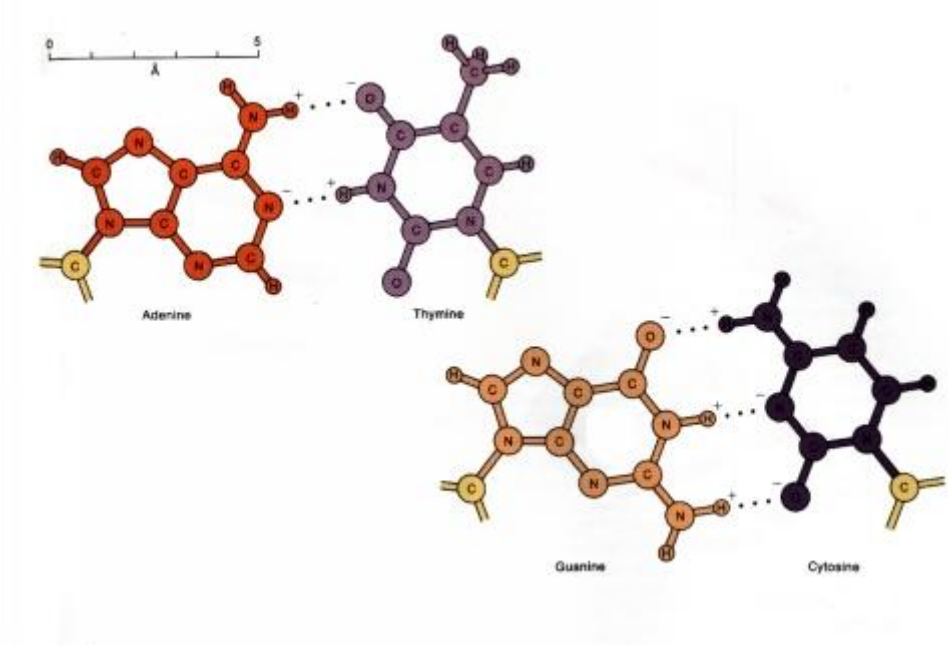


Figure 3: Base Pairing in DNA. Image courtesy of www.cse.ucsc.edu.

The four nucleotide constituents of DNA have in common a 5' phosphoryl and 3' hydroxyl group by reference to their positions in the deoxyribose sugar molecule. The formation of phosphate bonds between the 5' and 3' positions of the constituent nucleotides results in the DNA molecule, as exemplified in Figure 4.

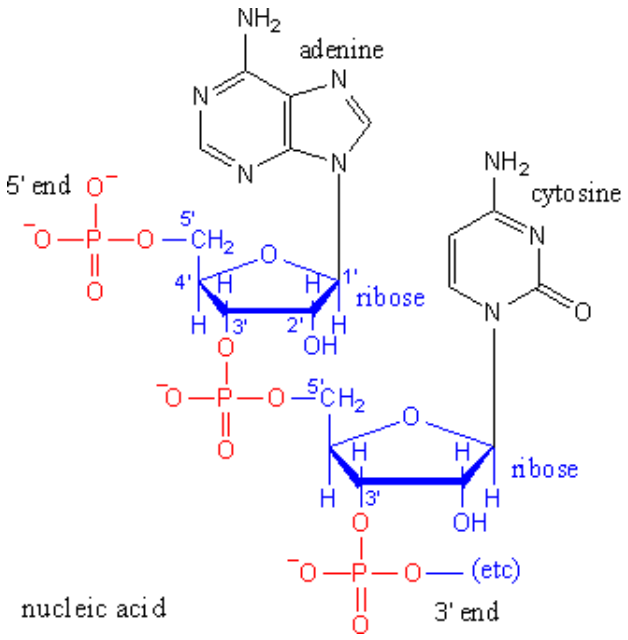


Figure 4: Phosphate bonding forming a polynucleotide.

2.5.2 The Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) first appeared in 1985 as a method for the prenatal diagnosis of sickle cell anaemia[42]. It was based on the remarkable insight of Kary Mullis, who realised that repetition of a DNA extension reaction bounded by two synthetic nucleotide primers would generate a large quantity of any specified DNA sequence[43].

Biosensory investigation of DNA requires that enough of the DNA under study is available for analysis. PCR exploits the natural function of polymerase enzymes which copy genetic material and proofread and correct the copies. PCR requires a template molecule- the DNA and two primer molecules to start the copying process. The primers are short chains of the four bases and they must be duplicates of nucleotide sequences on either side of the piece of DNA of interest. There are three basic processes involved in PCR, as exemplified in Figure 5.

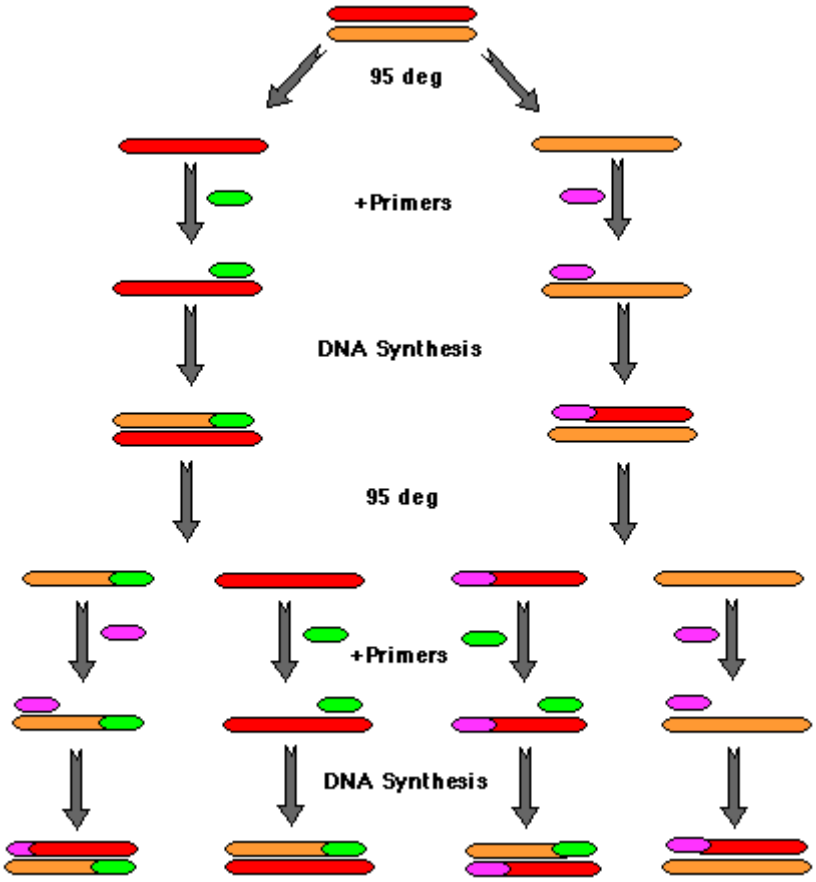


Figure 5: Polymerase Chain Reaction. Image courtesy of http://members.aol.com

Firstly, the double helix of the DNA must be denatured and separated by heating to 90-96^{o}C; a process called "melting". The second step is hybridisation or annealing, in which the primers bind to their complementary bases on the now single-stranded DNA. The annealing temperature (or melt temperature) is described as the temperature at which two strands of polynucleotides will chemically bind to form a duplex. As expected, annealing temperatures are specific to particular DNA sequences. The third is DNA synthesis by a polymerase. Starting from the primer, the polymerase can read a template strand and match it with complementary nucleotides. The result is two new helixes in place of the first, each composed of one of the original strands plus the newly assembled complementary strand. The amount of double stranded DNA produced increases exponentially and using commercially available equipment, millions of copies of a specific DNA strand can be obtained. The PCR process whereby each strand of the duplex is amplified as many times as its counterpart is referred to as "symmetric PCR" i.e., the same concentration of forward and reverse primer is utilised.

2.5.3 Human and Pathogenic Genome Characterisation

Completed in 2003, the Human Genome Project (HGP) was a 13-year project coordinated by the U.S. Department of Energy and the National Institutes of Health. The primary project goals were to identify all the approximately 20000-25000 genes in human DNA and to determine the sequences of the 3 billion chemical base pairs that make up human DNA[44].

Alongside the HGP, parallel projects have run in order to characterise pathogen genomics. In 1995, the first microbe sequencing project, *Haemophilus influenzae* was completed. Encouraged by the success of that initial effort, researchers have continued to sequence an impressive array of other medically important microbes. The National Institute of Allergy and Infectious Disease (NIAID) has made significant investments in large-scale sequencing projects and includes projects to sequence the complete genomes of many pathogens, including *C. trachomatis* as well as the bacteria that cause tuberculosis, gonorrhoea and cholera and organisms that are considered agents of bioterrorism[45].

2.5.4 DNA-based Assays

Nucleic acid amplification-based methods are now of prime importance for the diagnosis of chlamydial infections[46]. A number of commercial products using nucleic acid amplification technology are currently available[47]. These tests amplify the target nucleic acid, DNA or ribonucleic acid (RNA); or the probe after it has annealed to target nucleic acid. Such tests are generally more sensitive than liquid or solid phase hybridisation tests, which do not embody an amplification process[48] and are considerably more sensitive than culture or antigen detection methods[49].

The major targets for amplification-based tests against *C. trachomatis* are generally multiple-copy gene products, such as the cryptic chlamydial plasmid or ribosomal RNA. Starting from a multiple copy gene offers a clear starting advantage with respect to sensitivity. Initial nucleic acid amplification based tests were based on the detection of chlamydial DNA in clinical samples. The well documented PCR, e.g. the Roche Amplicor® PCR, uses selected primers, nucleotide triphosphates (NTPs) and

taq polymerase to amplify chlamydial DNA sequences, which are captured by hybridisation on oligonucleotide-coated microplates. The Roche Amplicor® PCR was the first commercial nucleic acid amplification test to be made available for the diagnosis of human chlamydial infections.

The more recent BDProbeTec®ET (Becton Dickinson, USA) *C. trachomatis* and *Neisseria gonorrhoeae* amplified DNA assay uses a novel isothermal strand displacement amplification (SDA) method coupled with fluorescent energy transfer (ET) measurement to detect amplified product as it is produced[50, 51].

In late 2006, a variant strain of *C. trachomatis* with a 377 base pair deletion in the cryptic plasmid was reported from Sweden[52]. Prevalence was as high as 65% in some regions of the country. Some commercial nucleic acid amplification tests (NAATs) use primers that detect this region of the cryptic plasmid and therefore cannot detect the variant. The identification of a variant strain at a high prevalence has caused widespread concern in countries that undertake substantial volumes of testing using these methods. A requirement for a novel assay able to determinine chlamydial strain variance is currently emerging in GUM[53]. To date there is no evidence of variant strains in England or Wales; however the HPA is conducting a survey to determine whether variants are present.

The main advantage of the nucleic acid amplification-based diagnosis for Chlamydia is that such methods combine unsurpassed sensitivity with good specificity. However, the greater sensitivity of these assays means that accidental contamination with amplified product (amplicon) is a problem of major importance for device design, laboratory workflow and personnel.

2.5.5 Fluorescent Detection Methods

The use of photolabile schemes, coupled with photochemical screeing techniques and combinatorial phosphoramidite chemistry, allows for the fabrication of ‘gene-chips’ for massively parallel detection of target DNA sequences[54]. Optical biosensors based on fluorescence are highly sensitive and arrays containing thousands of unique probe sequences have been constructed[55]. Owing to the sophisticated and expensive nature

of the instrumentation required, gene-chip technology is best suited for laboratory applications. Cases in which large numbers of genes or sequences need to be simultaneously sampled, as in transcription profiling or single nucleotide polymorphism (SNP) discovery, are well suited to gene chip analysis[56-59]. Clinical diagnostics do not generally require such large data accumulation. However, reliability and generality irrespective of sequence is required. Besides the cost and sophistication of the instrumentation, the inconsistent yields of target synthesis and labelling, as well as non-uniform rates of fluorophore photobleaching can result in accuracies lower than those required for patient diagnosis[60].

Surface plasmon resonance (SPR), reports changes in the refractive index of thin metal film substrates that occur on adsorption of the analyte and is suitable for target detection in an array-based format[61]. To achieve detection limits sufficient for a diagnostically useful signal, it is usually necessary to amplify the hybridisation signal by increasing the amount of material deposited at the metal film surface, either before or after target capture. As with fluorescence-based techniques, SPR systems can be complex and costly, rendering them generally more suitable for research applications.

Perhaps the most straightforward optical readout technology involves single-stranded DNA labelled with gold nanoparticles that simply change colour upon hybridisation of the target sequences[62]. Using photographic silver developing solutions, a 'scanometric' small array platform for DNA analysis at a flatbed scanner has been demonstrated using this technology with 100pM sensitivity[63]. This technology, offering simplicity and sensitivity, could provide a useful approach for clinical diagnostic development with optical readout.

2.5.6 Electrochemical DNA Sensors

Electrochemical methods are well suited for DNA diagnostics. Because electrochemical reactions give an electronic signal directly, there is no need for expensive signal transduction equipment. Moreover, because immobilised probe sequences can be readily confined to a variety of electrode substrates, detection can be accomplished with an inexpensive electrical analyser. Portable systems for clinical

testing and on-site environmental monitoring have been developed. Sensitive electrochemical signalling strategies based on the direct or catalysed oxidation of DNA bases, as well as the redox reactions of reporter molecules or enzymes recruited to the electrode surface by specific DNA probe-target interactions and by charge transport reactions mediated by the π-stacked base pairs have all been demonstrated[64]. Since Paleček discovered the electrochemical activity of nucleic acids[65] and sequentially, the systematic research in electrochemical DNA sensors started at the beginning of the 1990s, electrochemical DNA sensors were studied deeply and attracted considerable attention in clinical examination of inherited diseases and drug screening. Most of the electrochemical DNA biosensors are based on hybridization using the following procedures: 1) The ssDNA probe (or target) is immobilized onto a solid electrode surface. 2) The ssDNA probe (or target) is bound to its complementary strand presenting in a sample solution forming a double helix structure on the electrode surface. 3) A measurable electrochemical signal caused by an electro-active label or redox-active intercalator or the different electrochemical behavior of ssDNA and double-stranded DNA (dsDNA) after hybridization. An ideal immobilization method has to meet two requirements: 1) the electrode surface of the DNA biosensor allows effective and only specific binding of the ssDNA probe (or target) and (2) after the immobilization of ssDNA, the electrode surface remains electro-active for detecting DNA hybridization[66]. With the development of electrochemical DNA biosensors, there are effective methods reported for DNA immobilizing on electrode surface, such as electrostatic attraction, chemical adsorption, covalent-binding, antigen-antibody method[66], incorporating with a polymer matrix[66] and molecular self-assembly[66] on bare or chemically modified electrodes. The earliest and simplest DNA immobilization strategy was just dispersing DNA molecules on an electrode substrate until they were physically adsorbed onto the electrode surface. This method didn't require special chemical modification of the DNA or/and the electrode surface; therefore, it resulted in simplicity and ease of operation. However, the adherence of the DNA bases to the substrate led to an inaccessibility of the immobilized DNA during hybridization[66].

2.5.7 Array Sensors

Recently, a large number of designs for DNA-based electrochemical sensing have appeared. These types of sensors combine nucleic acid layers with electrochemical transducers to provide a biosensor and aim to provide a simple, accurate and inexpensive platform for patient diagnosis.

In a typical configuration, a single-stranded probe sequence is immobilised within the recognition layer, where base-pairing interactions recruit the target DNA to the surface. The repetitive, essentially uniform structure of DNA makes its assembly on the recognition surface well defined. It is at this interface that the critical dynamics of the target capture take place to generate the recognition signal; therefore, immobilising nucleic acid probe sequences in a predictable manner while maintaining their affinity for target DNA is crucial to overall device performance[67].

The earliest electrochemical DNA sensing strategy was based on reduction and oxidation of DNA at a mercury electrode. The amount of DNA reduced or oxidised reflected the amount of DNA captured. Palecek *et al.*[68] developed methods to discriminate single- against double-stranded DNA through direct DNA reduction. More recently, DNA oxidation has been carried out through adsorption stripping voltammetry (ASV)[69]. This technique achieves high sensitivity by inducing an electrostatic accumulation of analyte at the electrode surface before the detection step. The purine bases of DNA can be oxidised electrochemically, and this process can be carried out using carbon, gold, indium tin oxide (ITO) and polymer-coated electrodes[70].

Although this methodology is inherently sensitive, its application is complicated by significant background currents at the relatively high potentials required for direct DNA oxidation. Numerous methods to improve the signal-to-noise ratio have been developed, but more recent designs employ physical separation techniques to remove the sources of background interference. For example, Palecek *et al.*[71] and Wang *et al.*[72] have separately reported a two-step strategy for capturing target sequences using probe DNA immobilised onto magnetic beads. After target hybridisation, the beads are magnetically sepatated from the pool of analytes. The collected DNA is

depurinated in acidic solution, and the free guanine and adenine nucleosides are collected and analysed using ASV. As few as 40 femtomoles of substrate have been detected using this assay.

A similar technique using the direct guanine oxidation signal at carbon paste electrodes has recently been reported, in which specific genotypes of the factor V Leiden mutation in PCR amplicons were identified[73]. The use of peptide nucleic acid probes[74] affords more stringent control over hybridisation, and recent studies have shown that point mutations in target DNA can be more readily discerned using this methodology.

Methods to oxidise target DNA indirectly through the use of electrochemical mediators have also been explored. An interesting approach uses polypyridyl complexes of Ru(II) and Os(II) to mediate the electrochemical oxidation of guanine. Yang *et al.*[75] have used this method to detect trinucleotide-repeat expansions, in which catalytic currents due to the oxidation of guanine residues immobilised within the target sequence show a linear dependence on the repeat number. This same technique has been coupled to a reverse transcription-PCR assay to monitor the over-expression of genes in tumour samples[76]. Experiments with model PCR products have shown that the sensitivity of this system extends down to 550 attomoles of target DNA.

Several strategies have been pursued in which target DNA sequences are labelled with redox-active reporter molecules. Appearance of the characteristic electrochemical response of the redox reporter therefore signals the hybridisation event[77, 78]. A variation on this approach involves a three-component 'sandwich' assay, in which the redox label has been attached to a synthetic sequence specifically designed to bind to an overhang portion of the probe-target complex[79]. This dual hybridisation approach eliminates the need to modify the target strand, with the function of bringing together the probe and labelled sequences. In one example, ferrocene-labelled reporter strands signal the presence of target DNA hybridised to thiolated probe sequences immobilised onto gold electrodes. Incorporation of a second redox reporter (dimethylcarbamyl ferrocene), with an oxidation potential 170mV more positive than the ferrocene analogue, allows the detection of two targets simultaneously and

without spatial separation, much like a multicolour fluorescence labelling assay[80]. Using AC voltammetry, the detection of 50nM target concentrations and the identification of a GT single-base mismatch was accomplished.

Colloidal gold nanoparticles have also been employed to signal hybridisation in a sandwich-based assay. In one study, the labelled target is captured by probe strands immobilised on a graphite electrode, and hybridisation is detected electrochemically with the appearance of a characteristic gold oxidation signal[81]. The signal is greatly enhanced because of the large electrode surface area and the availability of many readily oxidised gold atoms in each nanoparticle label. The detection limit for PCR amplicons was found to be as low as 0.8 femtomoles of DNA.

Through the use of nanoparticle labels with different redox potentials, Wang *et al.*[82] have developed a technique in which these nanoparticles encode DNA sequences. Probe modified magnetic beads are hybridised with target DNA, separated magnetically from the pool of analytes and hybridised again with the nanoparticle-labelled reporter strands. The products are isolated, and the nanoparticles are dissolved and analysed by ASV. Electrochemical detection of three targets simultaneously was demonstrated with CdS, ZnS and PbS nanoparticle tags.

Many applications of DNA sensing involve extremely small numbers of target analytes, with correspondingly few hybridisation events. Analyte concentration may, as previously considered, be increased through the use of PCR, although in addition to this, in an effort to improve transduction of the hybridisation signal, an electrode surface may also be modified with a polymer layer that confers desirable properties, such as electrical conductivity, amenability to probe immobilisation or protection of the electrode from non-specific analyte adsorption. Heller *et al.*[83] have reported an enzyme-amplified DNA sensing technique involving the electropolymerisation of a polycationic redox polymer, upon which amine-terminated oligonucleotide single strands are electrodeposited by ligand exchange with bipyridyl osmium complexes impregnated within the polymer gel. Target DNA is captured at the electrode surface and subsequently hybridised to a reporter strand modified with horseradish peroxidase (HRP). This technique has been extended to screen-printed carbon electrodes on polyester sheets, which are inexpensive and amenable to mass-manufacture[84].

Biocatalysed production of insoluble products has been used by Willner *et al.*[85] to sense DNA hybridisation electrochemically at probe-modified electrodes. Target DNA is captured at a probe-modified gold electrode, where a redox-active DNA intercalator electroanalytically generates peroxide, which, in turn, is oxidised by HRP. The product precipitates onto the electrode, blocking the reaction of ferricyanide and providing the basis for DNA detection. In a variation on this approach, 5-bromo-4-chloro-3-indoyl phosphate is enzymatically converted to an insoluble indigo product, blocking ferricyanide; using this method, the detection limit for target DNA was extended down to $5x10^{-14}M$[86]. An enzyme-based hybridisation assay for detection of two DNA targets has also been described in which alkaline phosphatase and β-galactosidase are used to differentiate between two DNA targets by measuring the chronopotentiometry of their electroactive products[87]. These products yield well-resolved oxidation signals at carbon electrodes to allow amplified dual-target electrochemical detection.

In an alternative approach to chemical labelling schemes, redox-active reporter molecules that intrinsically associate with the double helix noncovalently have been successfully used for electrochemically based DNA analysis. In these analyses, rather than serving as a reactant, the DNA is the mediator. Such assays provide high sensitivity and simplicity.

The notion that electrochemical reactions could be used to signal DNA hybridisation was first explored by Milan *et al.*[88]. Their assay features a single-stranded probe sequence adsorbed onto glassy carbon, wherein hybridisation of target DNA caused and increase in the surface concentration of electrostatically bound $Co(phen)_3^{3+}$ as a result of the higher negative charge density at the hybridised surface. Characteristic redox reactions of the cobalt probe provided the electrochemical signal. More recently, Steel *et al.*[89] reported the effective use of $Ru(NH_3)_6^{3+}$ probe molecules to signal DNA hybridisation at gold films modified with thiol-bearing DNA probe sequences. As the DNA is captured to form double-stranded product, proportionally more ruthenium hexamine binds, yielding a higher signal.

Work by Barton *et al.*[90] has focussed on intercalative probe molecules; taking advantage of the electronic structure of double-helical DNA they have used, using intercalated redox probe molecules to report on perturbations in base stacking. The DNA base pair stack mediates charge transport to the intercalary bound at the top of the film. If the base pair stack is intact, the DNA mediates charge transport and current can flow. This chemistry has been found to be sensitive to DNA structure and perturbations in structure. Assays of DNA-mediated electrochemistry are therefore suited to sense changes in DNA: damage, mistakes, mismatches and even protein binding.

2.5.8 Real Time PCR

The real-time PCR system is based on the detection and quantification of a fluorescent reporter[91, 92]. The signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of nucleic acid target, the sooner a significant increase in fluorescence is observed[93].

A fixed fluorescence threshold is set significantly above the baseline that can be altered. The parameter C_T (threshold cycle) is defined as the cycle number at which the fluorescence emission exceeds a fixed threshold. There are three main fluorescence-monitoring systems for DNA amplification[94]: hydrolysis probes, hybridising probes and DNA-binding agents[95, 96]. Hydrolysis probes include TaqMan probes[97], molecular beacons[98-101] and scorpions[102- 104]. They use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target sequences in DNA samples.

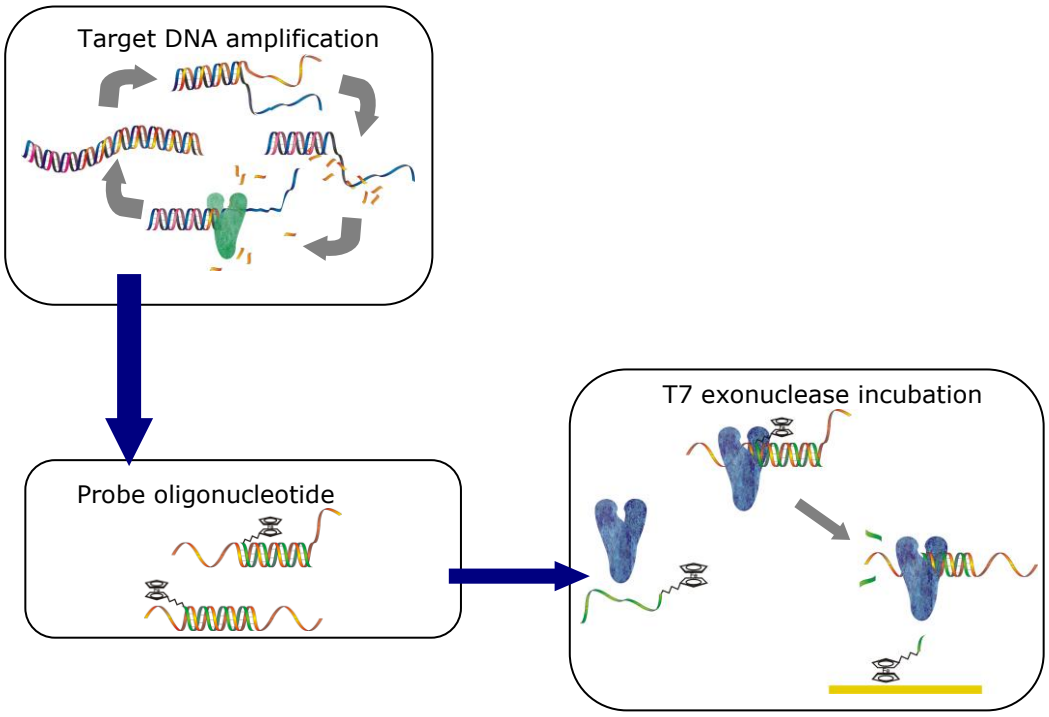
TaqMan probes are oligonucleotides longer than the primers (20-30 bases long with a double-strand melting temperature (Tm) value of 10^oC higher) that contain a fluorescent dye usually on the 5' base and a quenching dye (usually TAMRA) typically on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing (this is called FRET:

Förster or fluorescence resonance energy transfer)[105,106]. Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the probe[107]. This ends the activity of the quencher (no FRET) and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye. The TaqMan assay uses the universal thermal cycling parameters and PCR reaction conditions. Because the cleavage occurs only if the probe hybridises to the target, the origin of the detected fluorescence is specific amplification. The process of hybridisation and cleavage does not interfere with the exponential accumulation of the product. One specific requirement for fluorogenic probes is that there is no G at the 5'end. A G adjacent to the reporter dye quenches reporter fluorescence even after cleavage.

All real-time PCR chemistries allow detection of multiple DNA species (multiplexing) by designing each probe or beacon with a spectrally unique fluorescence/quencher pair as long as the platform is suitable for melting curve analysis if SYBR green (a fluorescent DNA stain) is used. By multiplexing, the targets and endogenous control can be amplified in a single tube.

2.6 Electrochemical Gene Sensing using T7 Exonuclease

Scheme 1 outlines the processes involved during the T7 Exonuclease assay.



Scheme 1: Outline of the T7 Exonuclease Assay.

Target DNA is amplified using symmetric PCR with appropriate primers designed for the amplification of the target sequence of interest. Upon target sequence amplification, T7 exonuclease and an electrochemically-active labelled DNA probe, specific to the amplified target sequence is introduced to the matrix and binding of the probe to the target sequence occurs. T7 Exonuclease acts in the 5' to 3' direction, catalyzing the removal of 5' mononucleotides from duplex DNA and thus the electrochemically-labelled 5’ mononucleotide is detectable at an electrode surface.

If there is no target DNA present in the matrix, neither PCR nor enzymatic probe digestion will occur and a significantly diminished electrochemical response is observed.

This project aims to facilitate biosensory evaluation of DNA sequences particular to *C trachomatis* and *N gonorrhoeae*; taking advantage of the sensitive, inexpensive and emerging electrochemical transduction technique. DNA amplification techniques will be employed in order to improve the analyte signal and the inherent specificity of PCR will be exploited also.

This thesis reports the development of a novel enzymatic electrochemical assay for the multiplex detection of *C. trachomatis* and *N. gonorrhoeae* with a validation internal control gene.

Chapter 3 - Label Design and Synthesis

Through the synthesis of a range of electrochemically active molecular labels for DNA hybridisation probes, the aim was to facilitate detection of a number of analytes within the same amplification matrix. The syntheses involved classic synthetic organic reactions including Wadsworth-Emmons olefination and amide couplings and whilst the synthetic procedures are presented herein in stages for simplicity, synthesis was performed in a continuous process (i.e. starting material through to label) in order to afford the oligonucleotide labels. Syntheses were, however, interrupted in order to analyse the potential electrochemistry of the compounds, as detailed in this chapter. Characterisation, therefore, will be shown for the full molecular labels, as detailed on page 45.

3.1 Synthesis of 5-Ferrocenylidene-2,2-dimethyl-1,3-dioxinane-4,6-dione (Compound 1) and 5-Ferrocenyl-2,2-dimethyl-[1,3]dioxane-4,6-dione (Compound 2)

Contemporary diagnostics often take advantage of biological / non-biological conjugates. Among this type of compounds, metal-bearing nucleosides are one of the most important representatives of molecular hybrids as they can function as electrochemical and photoluminescent labels for nucleic acids, infrared labels, radioactive metal isotope carriers, active centres of DNA-directed artificial chemical nucleases, and metal-bearing components for the construction of probes for DNA-mediated electron transfer[108].

Metal centres are usually attached to the nucleoside unit in the form of a metal complex, and complex-containing nucleoside conjugates are predominantly constructed by one of two major pathways: a) the synthesis of a chelator-containing nucleoside followed by metal complexation, and b) the synthesis of a functionalised nucleoside to which a metal complex can be conjugated, although in the majority of cases, chelators are used as vectors for metal ions. One of the few exceptions is metallocene-type complexes, of which the best known example is ferrocene. Only the second example of nucleoside-metallocene-type conjugates described thus far is the

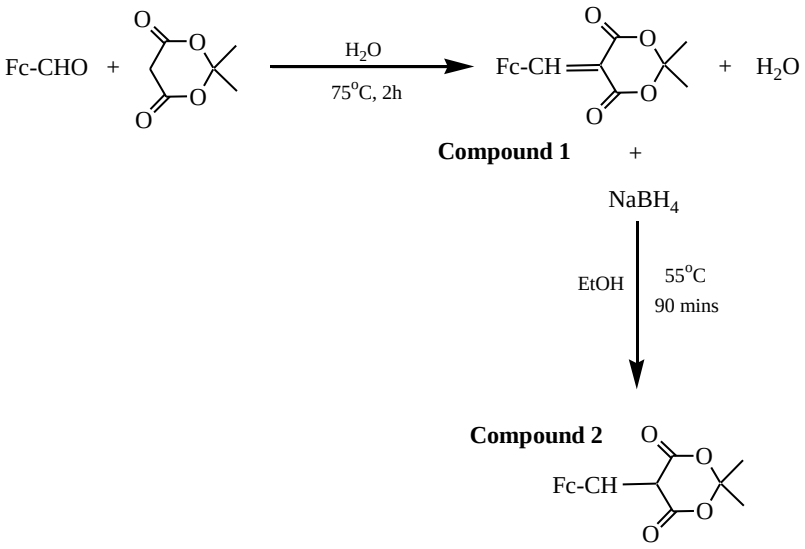
nucleoside-metallacarborane modifications reported recently by Olejniczak *et al.* [108] in which the metallocarborane modification is attached to the nucleoside component in a unique type of reaction between a dioxane-metallocarborane adduct and a base-activated nucleoside. This method, which was originally demonstrated for thymine nucleoside followed by incorporation of the obtained conjugate into a DNA-oligomer, can be considered as a third, direct pathway for the synthesis of metal-complex-containing nucleoside conjugates[108].

There has been thorough investigation into the factors affecting the redox potential of electrochemically active species, in particular, ferrocene. Silva *et al.*[109] studied the anodic behaviour of 34 ferrocene derivatives using cyclic voltammetry at at Pt electrode in an aprotic solvent. Electron withdrawing and donating behaviour of various substituents were evaluated through their Hammett σ_p and Taft polar σ^* constants and the half-wave oxidation potentials of the species were measured. It is evident from this study that an increase in the electron-withdrawing ability of the substituent, as measured by the Hammett σ_p constant, is associated with an increase in the half-wave oxidation potential of the species.

Silva's research later went on to report similar findings using disubstituted ferrocene moieties and proved successful in being predictive of a disubstituted species' anodic behaviour, using the Hammett constant σ_p of particular substituents[110].

Both studies indicated that the oxidation potential of ferrocene derivatives may be used as a probe in the study of the electronic properties of substituent groups, since their effects are transmitted through the carbocyclic ring to the iron redox active centre. It is therefore intuitive that the oxidation potential of a ferrocene derivative may be engineered through exploitation of the electronic properties of substituent groups.

An uncatalysed condensation reaction of 2,2-Dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid) with Ferrocene carboxaldehyde was performed according to part of a reported clean synthesis in water by Bigi *et al.*[111], to afford 5-Ferrocenylidene-2,2-dimethyl-1,3-dioxinane-4,6-dione (Compound 1), as shown below (Scheme 2).



Scheme 2: The Synthesis of Compounds 1 and 2.

Ferrocenecarboxaldehyde, as purchased from Lancaster Synthesis (Morecambe, Lancashire, UK.), (0.63g, 2.94mmol, 1.0 equiv) was added to a solution of Meldrum's acid from Sigma-Aldrich Ltd. (Gillingham, Dorset, UK.), (0.42g, 2.94mmol, 1.0 equiv) in Milli-Q 0.22μm filtered water (Millipore - Billerica, Massachusetts, USA), (100mL) at room temperature. The reaction mixture was allowed to stir for two hours at 75°C and the product was isolated by Buckner filtration, without further purification. Yield: 51.6%; R_f = 0.49 (ethyl acetate/hexanes, 7:3); *m/*z 340.156; ^{1}HNMR (300MHz, $CDCl_3$): 1.5(6H, s), 4.05(5H, s), 4.65(2H, m), 5.0(2H, m), 8.15(1H, s).

Compound 1 (0.50g, 1.46mmol, 1.0 equiv.) was dissolved in absolute ethanol, as purchased from Sigma -Aldrich. An excess of $NaBH_4$ (Sigma-Adrich) was added carefully at room temperature and the mixture was stirred for 90 minutes at 55°C. The reaction was allowed to cool to room temperature and subjected to an extractive workup using diethylether and saturated NaCl, both purchased from Sigma-Aldrich. The diethyl ether was removed *in vacuo,* yielding 5-Ferrocenyl-2,2-dimethyl-[1,3]dioxane-4,6-dione (Compound 2), as per Scheme 1. Yield 98%; R_f = 0.46 (ethyl acetate/hexanes, 7:3); *m/z* 338.140; ^{1}HNMR (300MHz, $CDCl_3$): 1.5(6H, s), 4.05(5H, s), 3.25(2H, s), 3.6(1H, s), 4.05(2H, m), 4.15 (5H, m), 4.25(2H, s).

3.2. Electrochemical Analysis of the Oxidation Potential of Organic Substrates

A 1mM solution of the compound was prepared in a phosphate buffered saline solution (PBS). The PBS was prepared by addition of one PBS tablet from Sigma-Aldrich Ltd. to 200mL of MilliQ filtered water, yielding 10mM phosphate buffer, 2.7mM potassium chloride and 0.137M sodium chloride. In order to aid solubility, the compound was firstly dissolved in 5mL of absolute ethanol from Fisher and hence made up to 50mL with the PBS, yielding a 10% Ethanol in PBS solvent.

A low volume cell (LVC) from Bioanalytical Systems Instruments Ltd. (BAS) (Kenilworth, Warwickshire, UK) was employed (Figure 6).

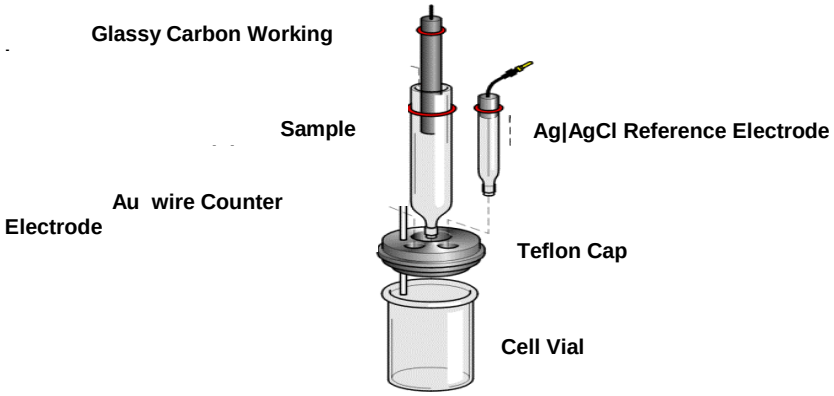


Figure 6: BAS Low Volume Cell. Image courtesy of BAS.

The LVC comprises of a freshly polished glassy carbon working electrode of 3mm in diameter, a gold wire counter electrode and a silver in 3M silver chloride reference electrode. The LVC was coupled to an Autolab potentiostat PGSTAT12, from Autolab Electrical Instruments (Utrecht, The Netherlands) and the system subsequently coupled to a PC for analysis using Autolab software.

With the cell vial and sample vial loaded with buffered solution and the electrodes in place (as per Figure 6), the differential pulse voltammogram was measured with the following potentiostat settings described in “Methods”.

3.2.1 Electochemical Characterisation of 5-Ferrocenylidene-2,2-dimethyl-1,3-dioxinane-4,6-dione (Compound 1) and 5-Ferrocenyl-2,2-dimethyl-[1,3]dioxane-4,6-dione (Compound 2)

It was envisaged that coupling ferrocene with Meldrum's acid would increase the oxidation potential of the iron centre, due to the electron withdrawing effect of the resultant conjugared system (as illustrated in Figure 7).

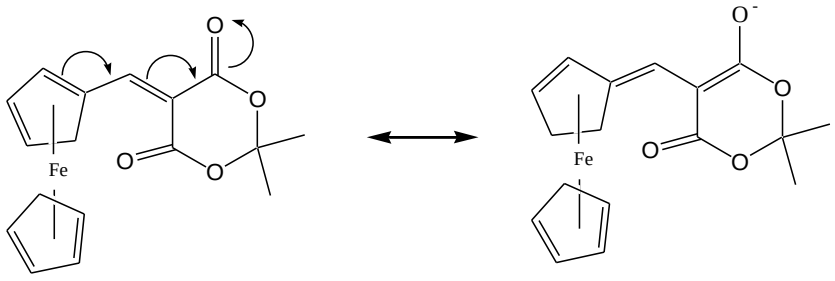


Figure 7: TheElectron Withdrawing Effect of Resonance on the Ferrocene Unit.

It was further envisaged that by simply reducing the alkene bond between the ferrocene moiety and the Meldrum's acid in 1, the electron withdrawing nature of the substituent would be lost as resonance would no longer be observed within the molecule, resulting in compound 2 (see Figure 8). Furthermore, it was believed that the alkyl substituent would have an electron donating effect upon the carbocyclic system, resulting in a lower oxidation potential of the iron centre with respect to ferrocene (220mV).

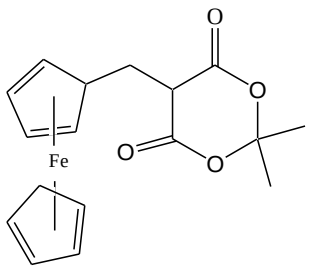


Figure 8: Loss of Rresonance by Reducing the alkene Between the Ferrocene Moiety and the Meldrum's Acid.

Figure 9 shows the differential pulse voltammogram of a 1mM solution of compound 1 in Phosphate Buffered Saline solution (PBS – inc. 10% EtOH).

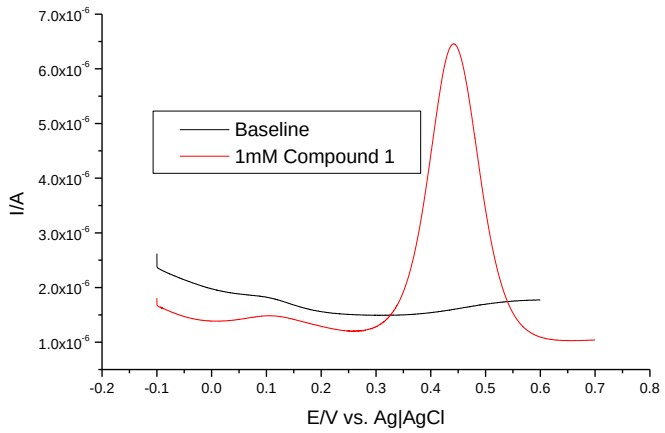


Figure 9: Differential Pulse Voltammogram of 1mM Compound 1 in PBS (inc. 10% EtOH)

Figure 10 shows the differential pulse voltammogram of a 1mM solution of compound 2 in PBS (inc. 10% EtOH).

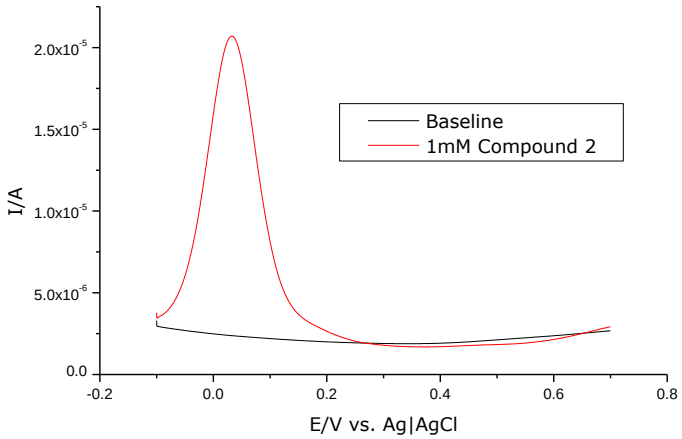
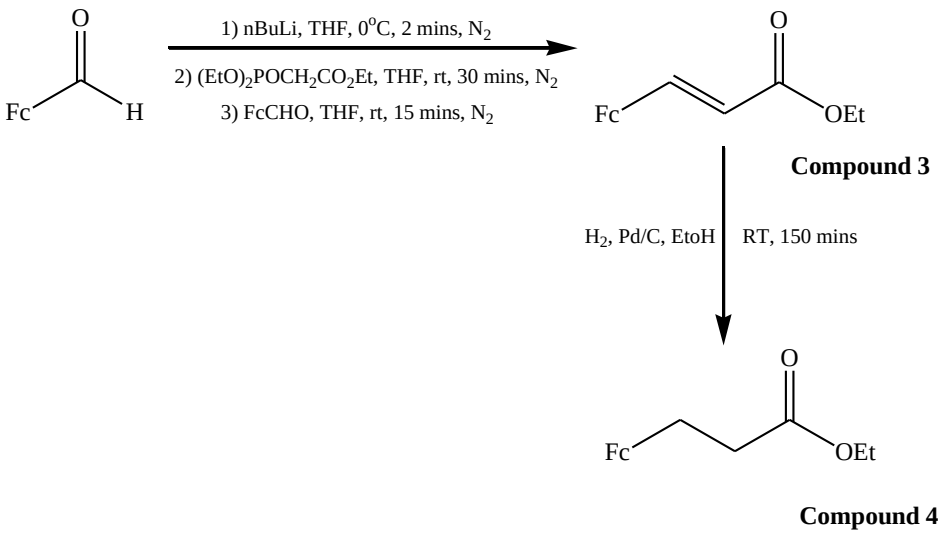


Figure 10: Differential Pulse Voltammogram of 1mM Compound 2 in PBS (inc. 10% EtOH).

The electron withdrawing nature of the conjugated system observed in compound 1 renders the ferrocene moiety of the molecule electron poor. This is reflected in an oxidation potential of the Fe(II)/Fe(III) redox couple of 0.45V. The loss of conjugation involved in compound 2, coupled with an electron donating effect from the alkyl substituent on the ferrocene, renders the ferrocene moiety of the molecule more electron-rich and thus more favourable to oxidation than compound 1; as reflected in the lower oxidation potential, with respect to ferrocene, of 0.05V.

3.3 Synthesis of (E)-3-Ferrocenyl-acrylic acid ethyl ester (Compound 3) and 3-Ferrocenyl-propionic acid ethyl ester (Compound 4)

The difference in electrochemical properties exhibited by compounds 1 and 2 render them potentially useful in a multiplex system owing to the difference in oxidation potential. In order to afford labels for DNA probes based on this behaviour, compound 3 was prepared via Wadsworth-Emmons olefination between ferrocenecarboxaldehyde and triethylphosphonoacetate. In order to afford compound 4, Compound 3 was subject to reduction using hydrogen with a palladium on activated carbon catalyst, purchased from Sigma-Aldrich Ltd, as illustrated in Scheme 3.



Scheme 3: Synthesis of Compounds 3 and 4.

Triethylphosphonoacetate (0.86mL, 4.22mmol), as purchased from Sigma-Aldrich, was dissolved in dry THF and added via a dropping funnel to a solution of nBuLi (Sigma-Aldrich) (1.69mL, 4.22mmol) in dry THF (25mL) at 0^{o}C under N_2. The reaction was then warmed to room temperature and allowed to mix for 30 minutes. Ferrocenecarboxaldehyde (0.78g, 3.52mmol) (Sigma-Aldrich) was dissolved in dry THF, which was subsequently added dropwise via a dropping funnel to the phosphonate ester anion solution. The reaction mixture was stirred for a further 15

minutes to allow for completion of the reaction. The solvent was removed *in vacuo* and the product was extracted using diethyl ether (30mL), as purchased from Sigma-Aldrich, and MilliQ filtered water (30mL). The aqueous layer was then re-extracted with diethyl ether (30mL). The organic fractions were combined and dried using anhydrous magnesium sulphate and the solvent was removed *in vacuo* to yield Compound 3 without further purification.

Compound 3 (500mg, 1.76 mmol) was dissolved in absolute Ethanol (20mL) and 10% catalytic amount of Palladium on activated carbon (0.19g, 0.18 mmol) was added. Hydrogen gas was then bubbled through the reaction mixture for five minutes and the mixture was allowed to stir for 150 minutes under a hydrogen atmosphere. The reaction mixture was filtered over Celite 521 filter agent from Sigma-Aldrich Ltd to afford Compound 4 without further purification.

3.3.1 Electochemical Characterisation of (E)-3-Ferrocenyl-acrylic acid ethyl ester (Compound 3) and 3-Ferrocenyl-propionic acid ethyl ester (Compound 4)

In order to confirm the feasibility of compounds 3 and 4 as label precursors for multiplex DNA detection, esters 3 and 4 were each pre-dissolved in EtOH and diluted to 1mM in PBS. The resultant solutions were then analysed using the BAS LVC.

The differential pulse voltammogram of a 1mM solution of compound 3 is displayed in Figure 11.

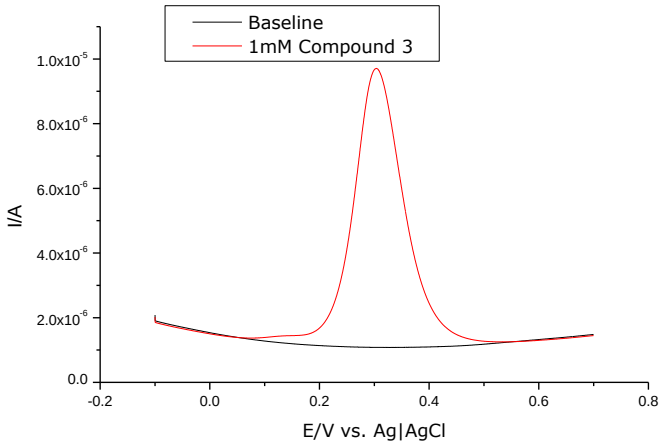


Figure 11: Differential Pulse Voltammogram of 1mM Compound 3 in PBS (inc. 10% EtOH).

The differential pulse voltammogram of a 1mM solution of compound 4 is displayed in Figure 12.

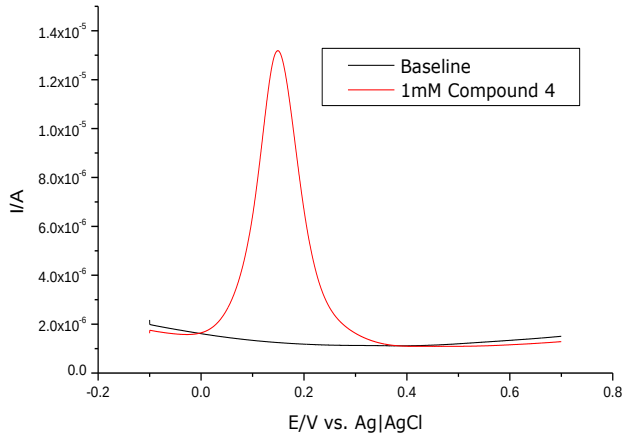


Figure 12: Differential Pulse Voltammogram of 1mM Compound 4 in PBS (inc. 10% EtOH).

The electron withdrawing nature of the conjugated system observed in compound 3 renders the ferrocene moiety of the molecule electron poor. This is reflected in an oxidation potential of the Fe(II)/Fe(III) redox couple of 0.3V. The loss of conjugation involved in compound 4, coupled with an electron donating effect from the alkyl substituent, renders the ferrocene moiety of the molecule more electron-rich and thus more favourable to oxidation than compound 1; as reflected in the lower oxidation potential of 0.15V.

In order to establish whether compounds 3 and 4 had potential to fulfil the multiplex objective with greater confidence, a solution of 1mM of each ester in 10% EtOH in PBS was analysed electrochemically. Figure 13 shows the differential pulse voltammogram of this multiplex solution.

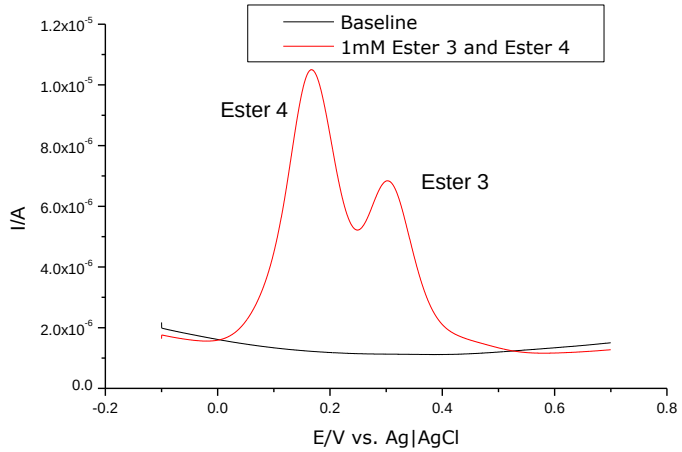
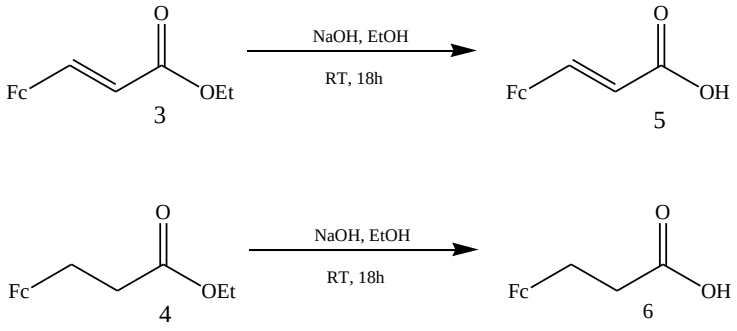


Figure 13: Differential Pulse Voltammogram of 1mM Compound 3 and 1mM Compound 4 in PBS (inc. 10% EtOH).

Clear differentiation between the two label precursors is observed, showing potential use in a multiplexing system. There is, however, an apparent decrease in the observed oxidation current for Ester 3, possibly due to instability of the alkene in the aqueous buffer together with the esters' partial insolubility in aqueous media.

3.4 Synthesis of (E)-3-Ferrocenyl-acrylic acid (Compound 5) and 3-Ferrocenyl-propionic acid (Compound 6)

In order to activate the compounds, rendering them accessible for amide linkage to a linker moiety and phosphoramidite terminus, Esters 3 and 4 underwent base hydrolysis using sodium hydroxide to afford carboxylic acids, as shown in scheme 4.

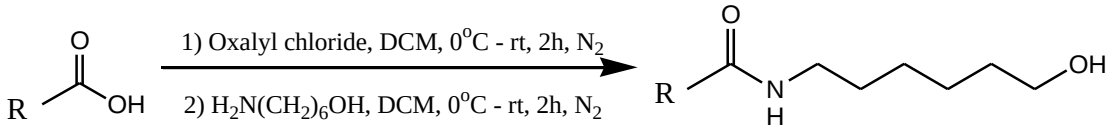


Scheme 4: Base Hydrolysis of Esters 3 and 4, yielding carboxylic acids 5 and 6.

To a solution of the ester 3 or 4 (1.3 mmol) in ethanol (25mL) was added NaOH (4.0mmol) (Sigma-Aldrich). The solution was heated to 50^{o}C and stirred for 18 hours. Acetic acid, as purchased from Sigma-Aldrich, was subsequently added to the reaction mixture to afford a solution pH of 6.5 (Fisherbrand Hydrus 300) and the product was extracted with DCM. The organic phase was washed with brine (3x20mL) and MilliQ filtered water (3x20mL) and dried over $MgSO_4$ (Sigma-Aldrich). The solvent was removed *in vacuo* to yield the product carboxylic acid 5 or 6; which was used without further purification.

3.5 Synthesis of (E)-*N*-(6-Hydroxy-hexyl)-3-ferrocenyl-acrylamide (Compound 7) and *N*-(6-Hydroxy-hexyl)-3-ferrocenyl-propionamide (Compound 8)

The acids prepared (Compounds 5 and 6) were converted to the corresponding acid chlorides and further coupled with aminohexanol to afford compounds 7 and 8. These compounds inherit an amide linker with an alcohol terminus available for further reaction with 2-cyanoethyldiisopropylchlorophosphoramidite; the attachment moiety for the 5' terminus of the oligonucleotide sequence of interest.



R = FcC_2H_2 5
R = FcC_2H_4 6

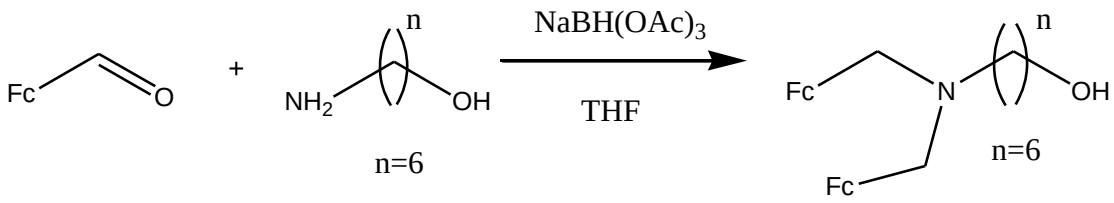
R = FcC_2H_2 7
R = FcC_2H_4 8

Scheme 5: Synthesis of 7 and 8.

Oxalyl chloride (10.32mmol), as purchased from Sigma-Aldrich, in dry DCM (2ml) was added dropwise, using a pressure equalising dropping funnel, to a stirred suspension of carboxylic acid 5 or 6 (4.3mmol) in dry DCM (100mL) at 0^{o}C under N_2. The reaction mixture was allowed to warm to room temperature and stirred for 2 hours. The solvent and remaining oxalyl chloride was carefully removed *in vacuo*. The acid chloride was then taken up in dry DCM (75mL) and 6-amino-hexan-1-ol (5.16mmol) (Sigma-Aldrich) in dry DCM (75mL) was added dropwise via a pressure equalised dropping funnel at 0^{o}C under N_2. The reaction mixture was stirred for two hours whilst warming to room temperature. The reaction mixture was washed with saturated $NaHCO_3$ (1x100mL), then 1.0M HCl (1x100mL). The organic layer was dried over $MgSO_4$ and the solvent removed *in vacuo*. The crude mixture was taken up in 50% EtOAC in petrol and filtered through a short silica pad in a sintered Büchner funnel capped with filter paper. The pad was then washed with 500mL of 50% EtOAc in petrol (Sigma-Aldrich) to remove any starting materials. The pure product was then eluted from the pad using EtOAc to give the amidoalcohol (7 or 8) as a powder.

3.5.1 Synthesis of 6-Diferrocenylamino-hexan-1-ol (Compound 9) and 6-Ferrocenylamino-hexan-1-ol (Compound 10)

In order to expand the potential range of phosphoramidite labels for use in the multiplex electrochemical system, a reductive amination of Ferrocenecarboxaldehyde using sodium triacetoxyborohydride was performed. A differocenyl label was prepared as per an established synthesis by Flower[112] (scheme 6) and a monoferrocenyl analogue was also synthesised to complement the range of monoferrocenyl labels already prepared.



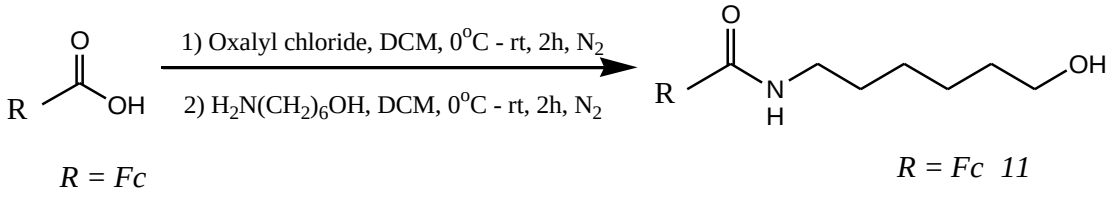
Scheme 6: Synthesis of 6-Diferrocenylamino-hexan-1-ol (Compound 9) and 6-Ferrocenylamino-hexan-1-ol (Compound 10)

Aminohexanol (1.00eq.) and sodium triacetoxyborohydride (3.00eq.) were added to Ferrocenecarboxaldehyde [(3.00eq.) for compound 9 (1.5eq.) for compound 10] in dry THF and the reaction mixture was stirred under N_2 for 12 hours at room temperature. 1M sodium hydroxide (4 eq. To $NaBH(OAc)_3$) and ethyl acetate of equal volume was added and the mixture was separated. The organic layer was washed with sat. $NaHCO_3$, sat. NaCl and H_2O and subsequently dried over $MgSO_4$. The solvent was removed *in vacuo*.

The crude product was purified *via* flash chromatography (SiO_2, 40% ethyl acetate, 60% petroleum ether (40-60^oC) +5% triethylamine.

3.5.2 Synthesis of *N*-(6-Hydroxy-hexyl)-ferrocenamide (Compound 11)

Compound 11 was synthesised as per Scheme 7 in order to compliment the range of phosphoramidite precursors already prepared (compounds 7-10).



R = Fc *R = Fc 11*

*Scheme 7: Synthesis of **N-(6-Hydroxy-hexyl)-ferrocenamide (Compound 11)***

Oxalyl chloride (10.32mmol) in dry DCM (2ml) was added dropwise, using a pressure equalising dropping funnel, to a stirred suspension of ferrocenecarboxylic acid in dry DCM (100mL) at 0^oC under N_2. The reaction mixture was allowed to warm to room temperature and stirred for 2 hours. The solvent and remaining oxalyl chloride was carefully removed *in vacuo*. The acid chloride was then taken up in dry DCM (75mL) and 6-amino-hexan-1-ol (5.16mmol) in dry DCM (75mL) was added dropwise via a pressure equalised dropping funnel at 0^oC under N_2. The reaction mixture was stirred for two hours whilst warming to room temperature. The reaction mixture was washed with saturated $NaHCO_3$ (1x100mL), then 1.0M HCl (1x100mL).

The organic layer was dried over $MgSO_4$ and the solvent removed *in vacuo*. The crude mixture was taken up in 50% EtOAC in petrol and filtered through a short silica pad in a sintered Büchner funnel capped with filter paper. The pad was then washed with 500mL of 50% EtOAc in petrol to remove any starting materials. The pure product was then eluted from the pad using EtOAc to give the amidoalcohol as a powder.

3.6 Synthesis of (Label JAOL1), (Label JAOL2), (Label JAOL3), (Label JAOL4) & (Label JAOL5)

Table 1 shows the corresponding Label name for an individual phosphoramidite precursor (Compound 7, 8, 9, 10 & 11).

Compound	Corresponding Label Name
7	LabelJAOL2
8	LabelJAOL3
9	LabelJAOL4
10	LabelJAOL5
11	LabelJAOL1

Table 1 – Corresponding Label Names

N,*N*-Diisopropylethylamine (8.4mmol) was added to a stirred solution of the amidoalcohol (**7, 8, 9, 10 or 11**) (2.1mmol) in dry THF (25mL) under nitrogen. 2-Cyanoethyldiisopropylchlorophosphoramidite (3.15 mmol) (Sigma-Aldrich) was added dropwise and the reaction stirred for 15 minutes. MilliQ filtered water (200µL) was added and the reaction stirred for a further 30 minutes. Ether – triethylamine (50/50, 25mL) (sigma-Aldrich) was added and a precipitate formed. The mixture was washed with saturated $NaHCO_3$ (25mL), then MilliQ filtered water (25mL). The organic layer was then dried over $MgSO_4$ and the solvent removed *in vacuo*. Purification by flash chromatography (SiO_2, 60% EtOAc/petrol + 1% Et_3N) gave the phosphoramidite (JAOL1, JAOL2, JAOL3, JAOL4 & JAOL5) as an oily liquid.

The phosphoramidites were stored at -20^oC, under nitrogen.

Label Characterisation

LabelJAOL1

Yield: 54%, Rf: 0.15 (ethylacetate/hexanes, 7:3), m/z: 529.45, ^{1}HNMR (300MHz, $CDCl_3$): 6.06(1H, br. m, NH), 4.66(2H, ap. S, H-cp), 4.27 (2H, ap.s, H-cp), 4.14(5H, s, H-cp'), 3.83(2H, dd, J 13.2, 6.9), 2.65(2H, t, 6.4), 1.56-1.00 (20H, m), ^{31}PNMR (121.5MHz, $CDCl_3$): 147.9 (s).

LabelJAOL2

Yield: 57%, Rf: 0.14 (ethylacetate/hexanes, 7:3), m/z: 559.5, ^{1}HNMR (300MHz, $CDCl_3$): 7.39(1H, d), 5.90(1H, d), 5.42(1Hbr. M, NH), 4.37(2H, ap.s, H-cp), 4.29(2H, ap.s, H-cp), 4.08(5H, s, H-cp'), 3.75(2H, ap. m), 3.55(2H, ap. m), 3.55(1H, ap. m), 3.26(2H, q), 2.57(2H, t), 1.35(20H, ap. m), ^{31}PNMR (300MHz, $CDCl_3$): 147.9 (s).

LabelJAOL3

Yield: 56%, Rf: 0.20 (ethylacetate/hexanes, 7:3), m/z: 557.5, ^{1}HNMR (300MHz, $CDCl_3$): 5.30(1H, br. M, NH), 4.03(5H, s, H-cp'), 3.97(2H, ap. m, H-cp), 3.75(2H, ap. m), 3.60(3H, ap. m), 3.15(2H, ap.q), 2.62(4H, m), 2.23(2H, t), 1.4(20H, ap. m), ^{31}PNMR (300MHz, $CDCl_3$): 147.9 (s).

LabelJAOL4

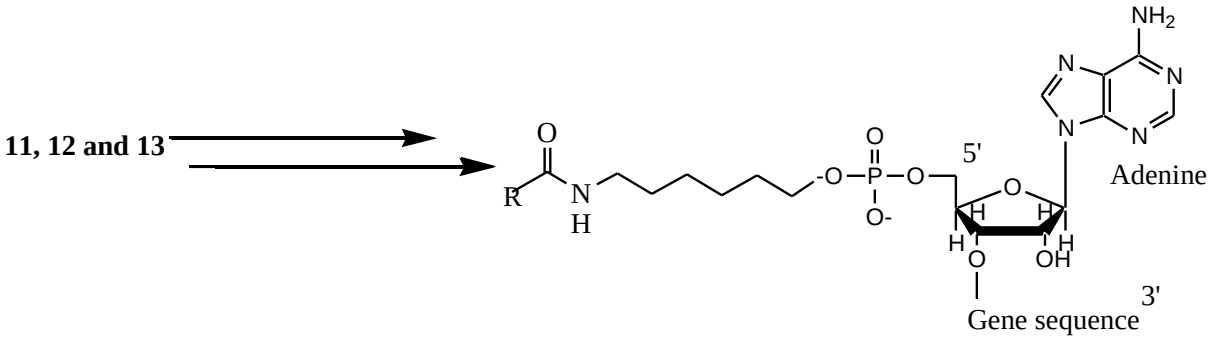
Yield: 54%, Rf: 0.7 (ethylacetate/hexanes, 7:3), m/z: 529.3, ^{1}HNMR (300MHz, $CDCl_3$): 6.06(1H, br.m, NH), 4.66 (2H, ap. s, H-cp), 4.27 (2H, ap. s, H-cp), 4.14 (5H, s, H-cp'), 3.83 (2H, m), 3.57 (4H, m), 3.28 (2H, dd, *J* 13.2), 2.65 (2H, t, *J* 6.4), 1.56-1.00 (20H, m), ^{31}PNMR (300MHz, $CDCl_3$): 147.9 (s).

LabelJAOL5

Yield: 37%, Rf: 0.8 (ethylacetate/hexanes, 7:3), m/z: 727.36, ^{1}HNMR (300MHz, $CDCl_3$): 4.1(4H, s), 4.05(4H, s), 4.01(8H, m), 3.7(1H, m), 3.5(2H, m), 3.3(1H, ap. q), 2.6(1H, ap. t), 1.55-1.00(20H, m), ^{31}PNMR (300MHz, $CDCl_3$): 147.9 (s).

3.7 Probe Manufacture – Probes JAOL1, JAOL2, JAOL3, JAOL4 & JAOL5

Labels JAOL1, JAOL2, JAOL3, JAOL4 and JAOL5 were then attached to the 5’ end of a specified oligonucleotide recognition sequence using standard solid phase coupling and reverse phase HPLC purification (atdBio, Southampton, UK), to afford labelled probe oligonucleotides, as exemplified in Scheme 8.



Scheme 8: Solid Phase Coupling of Phosphoramidite to Target DNA.

Labelling of synthetic oligonucleotides during solid-phase synthesis has the advantage that large quantities of labelled oligonucleotide can be prepared for major applications such as DNA diagnostics and forensic screening. The simplest way to label an oligonucleotide during solid-phase synthesis is to add the chemical modification to the 5’-end, although 3’-labelling and internal labelling is also possible using special phosphoramidite monomers. The addition of labels to amino- or thiol-modified oligonucleotides after solid-phase synthesis is less efficient and requires less care. Labelling a deoxy- or dideoxynucleoside triphosphate and incorporating the monomer using DNA polymerisation is an enzyme-catalysed procedure and is therefore a small scale method. However, it is a convenient technique for introducing a large number of labels into a DNA strand.

The labelled-oligonucleotide is further subject to reverse-phase HPLC purification.

Table 2 indicates the oxidation potential of each probe in PBS (including 10% EtOH) vs. Ag¦AgCl.

Probe Name	E_{ox} vs. Ag|AgCl (mV)
JAOL1	0.315
JAOL2	0.200
JAOL3	0.075
JAOL4	0.200
JAOL5	0.200

Table 2: Corresponding probe names to oxidation potential

Chapter 4 – Methods

4.1 – Differential Pulse Voltammetry

Throughout the scope of this thesis, all solutions of redox species contain background electrolyte in form of salts inherent in the buffers used and as such, the systems are described as being diffusion limited. The current-time response for the Faradaic process is described by the Cotrell equation (Equation 1):

$$|i| = \frac{nFA[O]\sqrt{D}}{\sqrt{\pi}\sqrt{t}}$$

Equation 1: The Cotrell Equation. n is the number of electrons, F is Faraday's constant, A is the electrode area, [O] is the bulk concentration of oxidised species, D is the diffusion constant.

Following a potential step, the Faradaic current decays at a rate proportional to $1/t^{1/2}$. Charging current, however, is also part of the total measured current and is proportional to e^{-t}. The Faradaic current, therefore, decays more slowly than the charging current. This is important, as the Faradaic current is directly proportional to the concentration of the electroactive ferrocene moiety (by the Cotrell equation – Equation 1), but the charging current is largely independent of the concentration of the electroactive species. In the systems under investigation, the concentrations of electroactive species are low, and as a result, the Faradaic current are also very low (nA – μA). The consequence of this is that the capacitive current may have distorted the measured values, leading to inaccuracies in the measurement. The solution to this problem is to use Differential Pulse Voltammetry.

Differential Pulse Voltammetry involves applying a potential step, waiting for some time (generally milliseconds), and then measuring the current. By waiting, the capacitive charging current will have decayed to very small amounts, but the Faradaic current will still be significant. In addition, the differential current is measured – that is the difference in current before the pulse and some time after the pulse. Thus, the application of a pulse sequence and the sampling of the current at some point before and at the end of the pulse can remove the effects of capacitive charging (Figure 14).

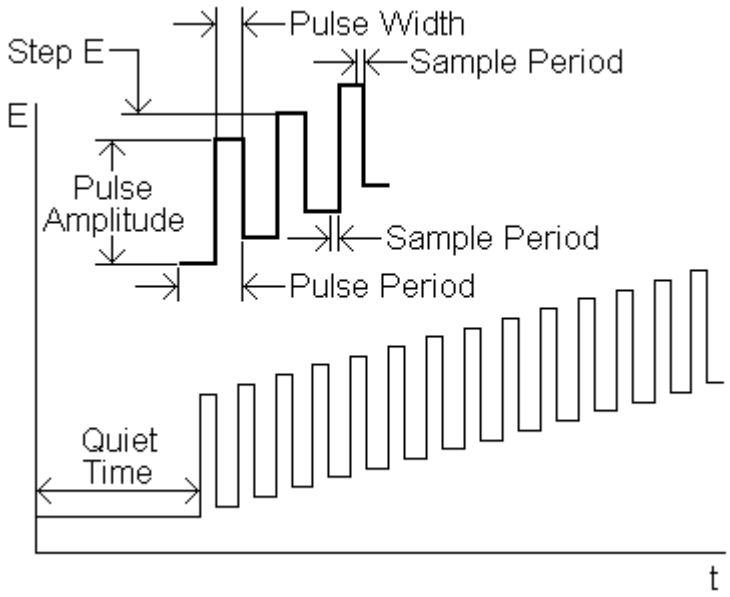


Figure 14: Representation of Differential Pulse sequence. E is potential (V) and t is time. Image courtesy of the University of Bath.

Throughout, the electrochemical cell was coupled to an Autolab potentiostat PGSTAT12, from Autolab Electrical Instruments (Utrecht, The Netherlands) and the entire system coupled to a PC for analysis.

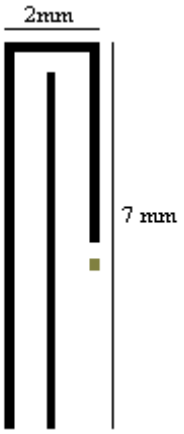
The potentiostat settings described below were adopted for all analyses unless specified) herein.

Pretreatment: *Conditioning potential (V): 0, Duration: 0s, Deposition potential: 0, Duration: 0, Equilibration time: 0,* ***Measurement:*** *Cell of after measurement:* X, *Modulation time(>=0.0025): .04, Interval time (>=0.105): .1,* ***Potentials:*** *Initial: -0.1, End: 0.5, Step: 0.003, Modulation amplitude: 0.04995, Standby potential (V): 0.*

3.2 – DPV Measurement, using Screen Printed Carbon Electrodes (SPCEs)

Electrochemical Analyses were performed on screen printed carbon electrodes (SPCEs), as purchased from Gwent Electronic materials (GEM) (Pontypool, Wales, UK), using the parameters detailed.

The screen-printing technology is widely used for the mass-production of disposable electrochemical sensors. The practical utility of screen-printed electrodes has been developed despite the fact that little is known about the nature of electrode reactions at these important microfabricated sensors[113]. The screen-printing microfabrication technology is commonly used for large-scale production of extremely inexpensive and yet highly reproducible electrochemical sensors [114-116]. Screen-printed sensors are expected to be widely used in numerous real-life applications of controlled potential techniques, and are already the devices of choice for the commercialisation of "one-shot" glucose[117] or metal[118] sensors.



The SPCEs used herein comprised of a central counter electrode (carbon graphite ink): 0.25mm X 6mm, an outer working electrode (carbon graphite ink): 0.5mm wide and the spacing between the electrodes was 0.375mm. The reference electrode comprised of a 0.5mm square (silver/ silver chloride paste).

The electrodes were manufactured on a Valox backing polymer; which has desirable inherent dielectric properties.

20µL samples for analysis were applied to the surface of the electrode, ensuring that the reference electrode, as well as the working and counter electrodes were covered. Owing to the hydrophobic interaction of the aqueous samples with the polymeric electrode coating, this often required manipulation of the sample using a sterile pipette tip.

4.2 – DNA Sequences

All sequences referred to herein are noted 5'-3'. Unless specified, all synthetic targets and primers were purchased from Sigma Genosys (Gillingham, Dorset, UK). All DNA probes were purchased from ATDBio (University of Southampton, UK).

N. gonorrhoeae

Synthetic Target:

TATTGTGTTGAAACACCGCCCGGAACCCGATATAATCCGCCCTTCAACATC
AGTGAAAATCTTTTTTTAAC

The red section is the recognition sequence for the hybridisation probe and the primer sequences are as follows:

Primer sequences:

NgF1 (Forward Primer): GTTGAAACACCGCCCGG

NgR1 (Reverse Primer): CGGTTTGACCGGTTAAAAAAAGAT

Genomic Target:

Neisseria gonorrhoeae genomic DNA was purchased from LGC Promochem (United Kingdom) and used at 10µg/mL.

C. trachomatis

The following sequences were identified for use by Atlas Genetics Ltd. and form part of the cryptic plasmid.

Synthetic Target:

CGCAGCTGCTGTAATCACCCAGTCGATAAATGTGTAAGCATACTTTGATG
CATTTGGGAAGCGCATTTTTATTTCTTAATATACATTTGCAGGCTTGATTA
CAAAGTAGGATTCTATTTGATCTACCAAGATAGGACATGGCTCTACAACG
AACCC

The red section is the recognition sequence for the hybridisation probe and the primer sequences are as follows:

Primer Sequences:
CHF3 (Forward Primer): GGGTTCGTTGTAGAGCCATGT
CHR4 (Reverse Primer): CGCAGCTGCTGTAATCACC

Genomic Target:
Chlamydia trachomatis E (BOUR Strain) Elementary Bodies were used at a concentration of ~8x10^7 EB's/mL, as purchased from USBiological (Massachusetts, USA).

β-Actin

The following sequences were identified for use by Atlas Genetics Ltd. and form part of the human β-actin gene.

Synthetic Target:
CCAGCCAGGTCCAGACGCAGGATGGCATGGGGGAGGGCATACCCCTCGT
A

The red section is the recognition sequence for the hybridisation probe and the primer sequences are as follows:

Primer Sequences:
BAF3 (Forward Primer): TGAAGCTGTAGCCGCGCTCGGT
BA4 (Reverse Primer): TCACCCACACTGTGCCCATCTACGA

Genomic Target:
A synthetic target was used for genomic assays, as purchased from Sigma Genosys.

4.3 – Amplification of Gene Targets using PCR

4.4.1: Uniplex PCR

For a uniplex PCR (one that amplifies a single DNA sectional sequence), the following generic PCR 'recipe' outlined below was applied.

Reagent	*Volume (µL)*
10xPCR Buffer (GE – Chalfont St Giles, Buckingham, UK)	2
1.5mMdNTPs (Promega – Southampton, UK)	2
10µM Primer Forward (Sigma Genosys – Gillingham, Dorset, UK)	1
10µM Primer Reverse (Sigma Genosys)	1
5000UmL^{-1}Taq Polymerase (GE)	0.2
25mM $MgCl_2$ (GE)	1.2
Target DNA	2
H_2O (Sigma Aldrich – Gillingham, Dorset, UK)	10.6
Total	20

Table 3: Reagent Volumes for a uniplex PCR

Using a DNA Engine from MJ Research (now Bio-Rad) (Hemel-Hempstead, Hertfordshire), with the following cycling parameters were employed to amplify the target DNA:

1=94.0^{o}C for 1:00
2=94.0^{o}C for 0:20
3=60.0^{o}C for 0:20
4=72.0^{o}C for 0:20
5=GOTO2, 39 times
6=72.0^{o}C for 3:00
7=4.0^{o}C for ever
8=END
This cycling program shall herein be referred to as "BACTIN3".

4.4.2 Duplex PCR

For a duplex PCR (one that amplifies two DNA sectional sequences), the following generic PCR 'recipe' outlined below was applied.

Reagent	*Volume (µL)*
10xPCR Buffer (GE – Chalfont St Giles, Buckingham, UK)	2
1.5mMdNTPs (Promega – Southampton, UK)	2
10µM Primer $F_{Sequence\ 1}$ (Sigma Genosys – Gillingham, Dorset, UK)	1
10µM Primer $R_{Sequence\ 1}$ (Sigma Genosys)	1
10µM Primer $F_{Sequence\ 2}$ (Sigma Genosys)	1
10µM Primer $R_{Sequence\ 2}$ (Sigma Genosys)	1
5000UmL^{-1}Taq Polymerase (GE)	0.2
25mM $MgCl_2$ (GE)	1.2
Sequence 1 Target DNA	2
Sequence 2 Target DNA	2
H_2O (Sigma Aldrich – Gillingham, Dorset, UK)	6.6
Total	20

Table 4: Reagent Volumes for Duplex PCR

Thermal cycle BACTIN3 was used to amplify the target sequences.

4.4.3 Triplex PCR

For a triplex PCR (one that amplifies three DNA sectional sequences), the following generic PCR 'recipe' outlined below was applied.

	Chlamydia	**Gonorrhoea**	**β-actin**	**Triplex**
Reagent	*Volume (µL)*	*Volume (µL)*	*Volume (µL)*	*Volume (µL)*
10xPCR Buffer (GE – Chalfont St Giles, Buckingham, UK)	2	2	2	2
1.5mMdNTPs (Promega – Southampton, UK)	2	2	2	2
10µM Primer F_{CHL} (Sigma Genosys – Gillingham, Dorset, UK)	1	1	1	1
10µM Primer R_{CHL} (Sigma Genosys)	1	1	1	1
10µM Primer F_{NG} (Sigma Genosys)	1	1	1	1
10µM Primer R_{NG} (Sigma Genosys)	1	1	1	1
10µM Primer $F_{\beta\text{-}A}$ (Sigma Genosys)	1	1	1	1
10µM Primer $R_{\beta\text{-}A}$ (Sigma Genosys)	1	1	1	1
5000UmL^{-1}Taq Polymerase (GE)	0.2	0.2	0.2	0.2
25mM $MgCl_2$ (GE)	1.2	1.2	1.2	1.2
$8.5x10^{-7}$ EBmL^{-1} DNA_{CHL} (US Biological – Swampscott, Massachusetts, USA)	2	0	0	2
10µgmL^{-1} DNA_{NG} (ATCC – Manassas, VA, USA)	0	2	0	2
10nM $DNA_{\beta\text{-}Actin}$ (Sigma Genosys)	0	0	2	2
H_2O (Sigma Aldrich – Gillingham, Dorset, UK)	6.8	6.8	6.8	2.6
Total	20	20	20	20

Table 5: Reagent Volumes for Individual and Triplex PCR of Genomic Targets: Chlamydia trachomatis, Neisseria gonorrhoeae and Human β-actin.

Thermal cycle BACTIN3 was used to amplify the target sequences.

4.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method used in biochemistry and molecular biology to separate DNA, or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate further than longer ones[125] . All gels herein were prepared by dissolving agarose (Sigma-Aldrich) under microwave heat in 0.5% TBE (Tris/Borate/EDTA) buffer at 3% w/v. To 100mL of the resultant gel solution, 10µL of 10mg/mL ethidium bromide was added. The resultant mixture was poured into a gel tank with gel combs in place to produce wells upon setting. Once the gel had set, 10µL of the PCR product was added to 2µL of 6x Loading dye (Promega) and the PCR product was introduced into the wells and the gel was subject to 100V potential difference in 0.5x TBE.

4.5 UV Visualisation of gel product

The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is ethidium bromide, usually abbreviated as EtBr. It fluoresces under UV light when intercalated into DNA (or RNA). By running DNA through an EtBr-treated gel and visualizing it with UV light, any band containing more than ~20ng DNA becomes distinctly visible and an image can be taken. This procedure for gel visualisation is used herein.

4.6 T7 Exonuclease Electrochemical assay using amplicon product

To 20µL of the PCR product, 1µL of each probe [CHL3 (75mV), Ng14-151(1:10) (200mV) and BA1(315mV)] was added, followed by 2µL of T7 exonuclease and the matrix incubated as per "Inc-J program" (37^{o}C for 20 minutes, followed by cooling to 16^{o}C). The sample was then loaded onto a SPCE for analysis and electrochemical measurement was subsequently performed.

4.7 DNA Amplification from Clinical Samples

Clinical samples were obtained from Dr. Clare Ling, Dept. Virology at the Royal Free Hospital in London, UK. The samples were anonymous discard samples, which had been tested using a culture assay for *Neisseria gonorrhoeae* and using the BD Probetec^{TM} protocol for *Chlamydia trachomatis*. The *BD ProbeTec Chlamydia trachomatis* amplified DNA assay is based on the simultaneous amplification and detection of target DNA using amplification primers and a fluorescently labelled detector probe. The strand displacement amplification (SDA) reagents are dried in two separate disposable microwell strips. The processed sample is added to the Priming Microwell which contains the amplification primers, fluorescently labelled detector probe, and other reagents necessary for amplification. After incubation, the reaction mixture is transferred to the Amplification Microwell, which contains two enzymes (a DNA polymerase and a restriction endonuclease) necessary for SDA. The Amplification Microwells are sealed to prevent contamination and then incubated in a thermally controlled fluorescent reader which monitors each reaction for the generation of amplified products. The presence or absence of CT is determined by relating the *BD ProbeTec* MOTA (Method Other Than Acceleration) scores for the sample to pre-determined cut-off values. The MOTA score is a metric used to assess the magnitude of signal generated as a result of the reaction.

The patient samples provided were 8 tested negatives for *C. trachomatis* and 6 tested positives for *C. trachomatis*. All patients, however, were confirmed as negative for *N. gonorrhoeae*. Using the *BD ProbeTec* protocol, patient samples, whether they are urine or in a swab elution buffer, were initially centrifuged for thirty minutes at 2000 g and the supernatant carefully decanted in order to minimize any inhibition of the amplification stage. The samples were then lysed at 114^{o}C. Of each patient, two sub-samples were provided, one lysed and one unlysed. The lysed samples in each case, were used in the assay in order to improve amplification.

Table 6 indicates patient numbers for the samples provided, including the result of their analysis at the Royal Free Hospital. It should be noted that the assay was performed ‘blind’. It was not until later discussion with the RFH that the veracity of our assay was established.

Samples Typed Negative for *C. trachomatis*	07M195165 07M195167 07M195175 07M195172 07M195180 07M195179 07M195178 07M195176
Samples Typed Positive for *C. trachomatis*	07M194999 07M195129 07M195831 07M195139 07M195123 07M195149

Table 6: Sample Identification

PCR was performed using the recipe described below (Table 7). BACTIN3 was the amplification program used and PCR negative controls were performed using sigma water in lieu of clinical sample.

	Triplex
Reagent	*Volume (µL)*
10xPCR Buffer (GE – Chalfont St Giles, Buckingham, UK)	3
1.5mMdNTPs (Promega – Southampton, UK)	3
10µM Primer F_{CHL} (Sigma Genosys – Gillingham, Dorset, UK)	0.3
10µM Primer R_{CHL} (Sigma Genosys)	0.3
5000UmL^{-1}Taq Polymerase (GE)	0.3
25mM $MgCl_2$ (GE)	1.8
BD Probec Sample (Royal Free Hospital – London, UK)	2
H_2O (Sigma Aldrich – Gillingham, Dorset, UK)	19.3
Total	30

Table 7: PCR Components for Clinical Sample PCR

4.8 T7 Exonuclease Electrochemical assay using amplicon from clinical sample PCR

The PCR product from the methodology described in section 4.7 was subject to agarose gel electrophoresis and UV visualisation and electrochemical analyses were performed as per sections 4.4 and 4.5.

Chapter 5 - Electrochemical Mechanisms of Assay Discrimination

5.1: DNA adsorption to Electrode Surfaces

DNA adsorption is fundamental to many biological functions, notably in human pathology[126]. Adsorption occurs via a number of pathways, though primarily through electrodiffusive processes. Recent research has investigated rate-limiting steps of DNA adsorption and the factors affecting it. Nguyen *et al*., for example, have investigated the role of divalent cations in plasmid DNA adsorption to natural organic matter-coated silica surfaces[126].

DNA adsorption to inorganic surfaces, notably electrodes, has also been the focus of intense research. Xu *et al.*modified ss-DNA and ds-DNA onto the surface of two silanized indium tin oxide (ITO) electrodes through means of adsorption, respectively, and were found to have gained high concentration after being electrochemically characterised. The electrode, modified by ss-DNA, recognised the complementary ss-DNA in solution with good repeatability[121]. Much work has also been invested in researching techniques for investigating oxidative damage to DNA at electrode surfaces. Ferapontova *et al.* studied electrochemically induced oxidative damage to DNA with ds calf thymus DNA immobilized directly on a gold electrode surface via adsorptive processes[121].

With electrochemistry at phase boundaries becoming more interesting to research endeavours, Osakai *et al.* have recently exploited adsorbed DNA on a polarized 1,2-dichloroethane/water interface as a facilitator for cationic-surfactant transfer. The voltammetric behaviour of the DNA was investigated and the peak current obtained showed a Langmuir-type dependence on the DNA[122].

5.2: Differential Pulse Analysis

The electrochemical assay described herein is configured to yield a peak current for an electrochemically labelled moiety. As stated in the Introductory chapter, the

labelled moiety released post T7 exonuclease digest exhibits a higher peak oxidation current than an undigested probe.

Figure 16 displays the differential pulse voltammogram (DPV) response for a fully complementary probe – target sequence for Chlamydia (Methods), labelled with Probe JAOL3 (Label Design and Synthesis). A clear difference In peak current between the probe-target + T7 exonuclease and the two control measurements: complementary probe-target with heat deactivated T7 exonuclease and probe without target is clearly observed.

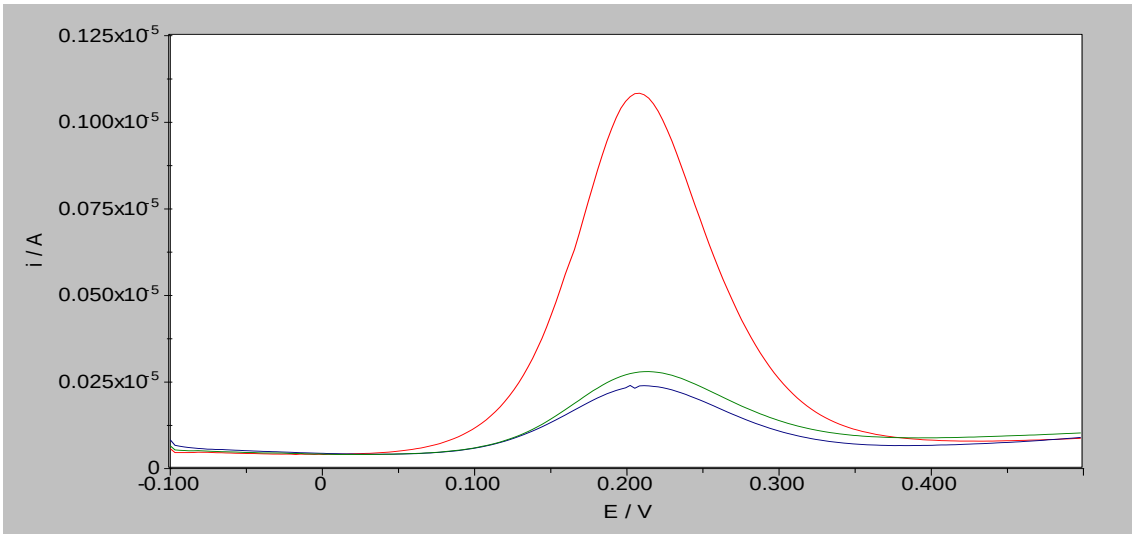


Figure 16: Chlamydia Exonuclease Assay

The basis of the observed discrimination between the peak oxidation currents for the probe-target + T7 and probe-no target + T7 exonuclease matrices was initially attributed to differences in diffusion coefficient of the enzymatically-digested electrochemically-labelled moiety and the labelled oligonucleotide probe. Further investigation, however, suggests a more subtle explanation for the observed behaviour.

5.3: Mass Spectral Analysis

A fundamental point in gaining better understanding of the mechanism of assay discrimination is the elucidation of the structure of the digest product released and detected at the electrode.

The mechanism of action of the gene 6 exonuclease of bacteriophage T7 has been thoroughly investigated[123] The enzyme hydrolyzes duplex DNA until about 50% of the DNA is classed as acid soluble. The remaining acid-insoluble DNA is single stranded and the acid-soluble product is almost entirely 5'-mononucleotides. The enzyme begins its exonucleolytic attack at the 5' terminus of DNA and hydrolyzes 5'-phosphoryl terminated and 5'-hydroxyl terminated DNA at equal rates. The exonuclease releases a dinucleoside monophosphate from a 5'-hydroxyl terminus and is able to release from one chain of DNA and begin hydrolysis of another before completion of hydrolysis. The enzyme is able to initiate hydrolysis at nicks as well as at external termini[123].

Figure 17, below, illustrates the T7 exonuclease assay used within the scope of this thesis.

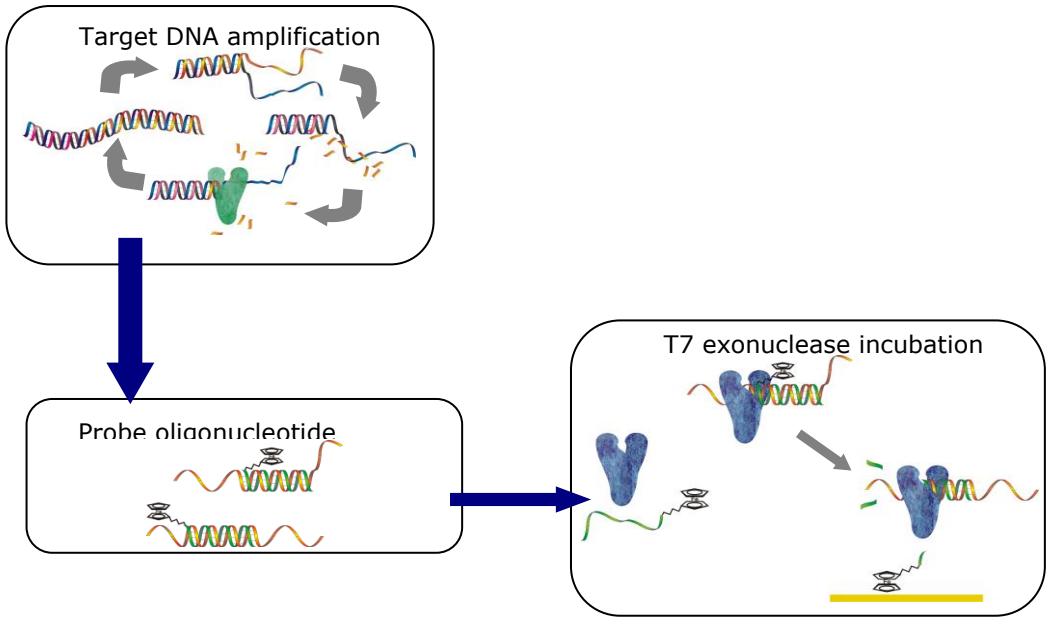


Figure 17: Outline of the T7 Exonuclease Assay

In the research carried out and described here, the 5' terminus of the probe duplex in this assay possessed a ferrocene-based moiety, mass-spectral analyses were performed in order to elucidate the composition of the digest fragments produced post-digestion in the enzymatic assay. The identifier sequence of the CTR gene (20 bases) for *Neisseria meningitides* was selected as the candidate for this study and the digest performed as described in "Methods". Label JAOL4 was attached to not only the probe sequence in this case, but to a separate mononucleotide, on the 5' base of the probe sequence (A) (as illustrated in Table 8) and to a separate base dinucleotide, on the two 5' bases of the probe sequence (AT).

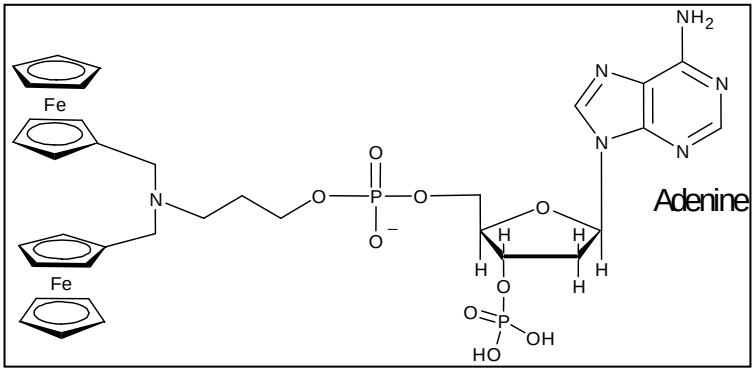


Table 8: Base Labelling of Label JAOL4.

The labeled mononucleotide and dinucleotide were hence used as molecular markers for the two potential digest fragments of T7 exonuclease. The analyses were performed on a micrOTOF mass spectrometer, using Bruker Daltronics data anaysis. Figure 18 displays the spectrum for the labelled mononucleotide and dinucleotide, each exhibiting peaks at their theoretical masses; $[M-H]^-$=863 and $[M-H]^-$=1167 repectively.

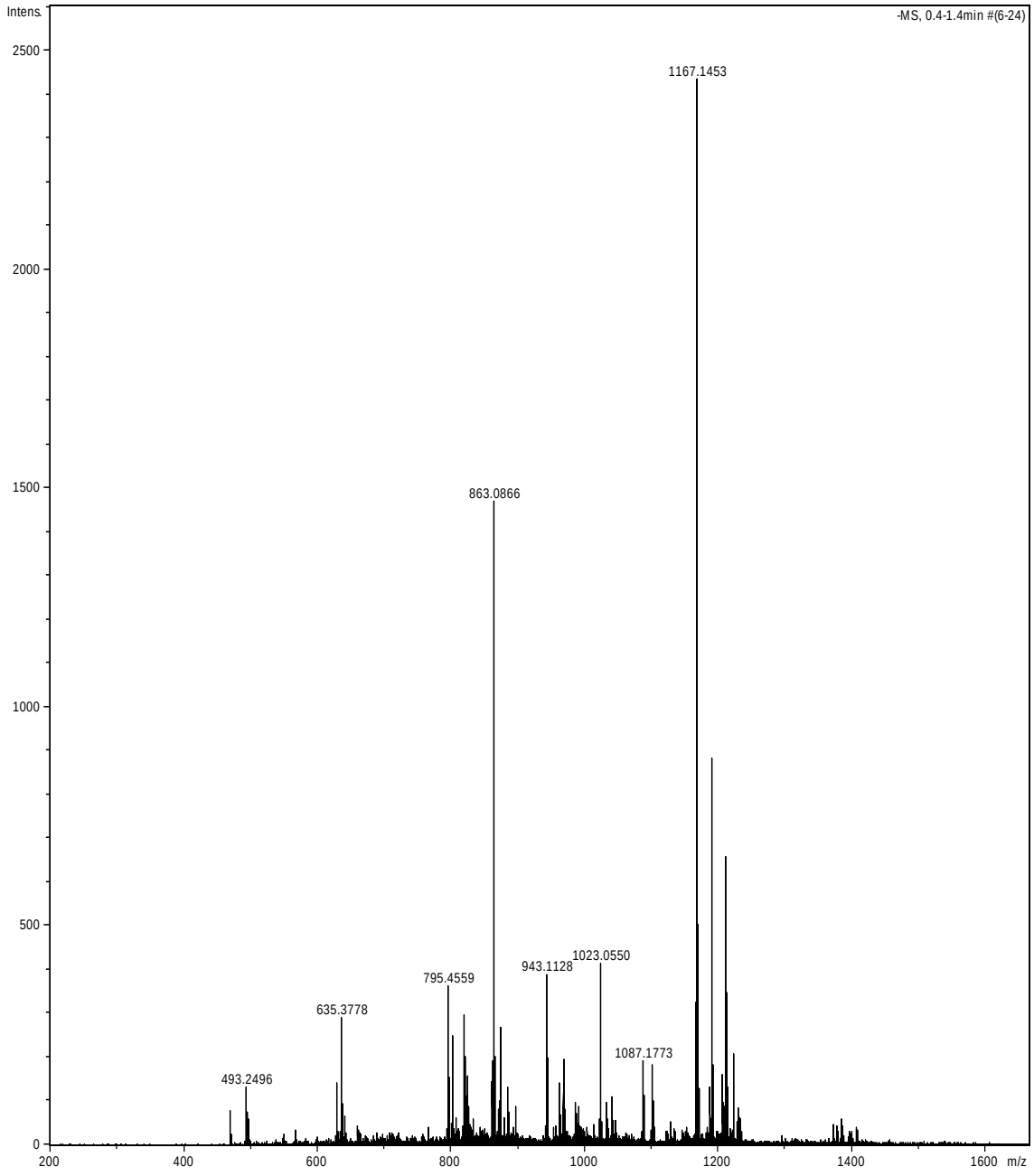


Figure 18: Mass Spectral analysis of the synthesised markers

The CTR gene probe and its complementary target were incubated with T7 exonuclease, and a mass-spectral analysis of the post-digest mixture was performed without further purification. The result of this analysis is displayed in Figure 19.

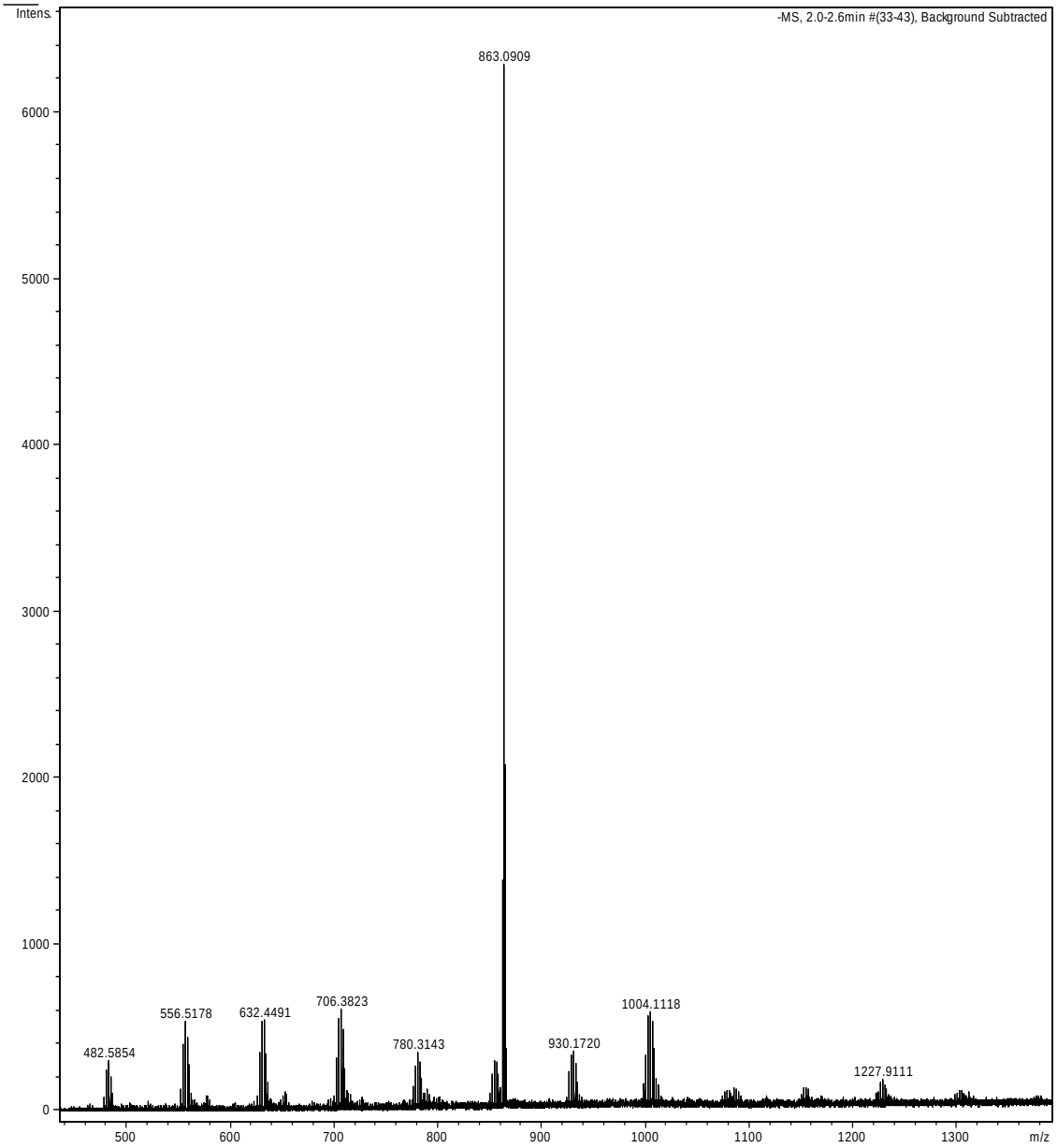


Figure 19: Mass Spectral analysis of the digest product

The single peak at m\z = 863 $[M-H]^-$ confirms that the electrochemical moiety, released to the electrode is in fact the labeled mononucleotide, with the enzymatic release of no labeled dinucleotide upon incubation of the assay matrix.

Figure 20 shows the DPV response for a fully complementary probe – target sequence for *N. gonorrhoea* labelled with probe BA probe, JAO2. The clear difference in peak current between the complementary probe-target and the probe without target can be clearly observed. The DPV response of a single nucleotide labelled with the same ferrocene label as in figure 20 was measured and is shown in figure 21, with a very

similar response being measured. Mass spectrometry of the T7 digested probe-target sequence also confirmed that a single nucleotide ferrocene was released. Evidently the electrode discriminates between a ferrocene labelled mononucleotide and the ferrocene labelled oligonucleotiode. The question that remains is what is the mechanism of discrimination?

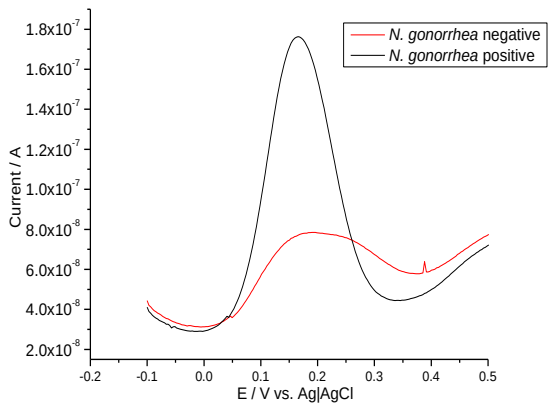


Figure 20: *Neisseria gonorrhoeae* digest

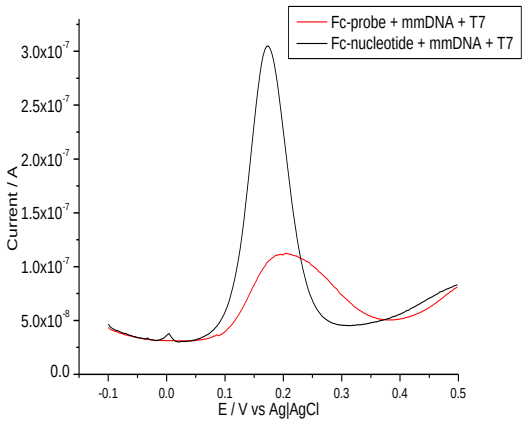


Figure 21: Single nucleotide labelled

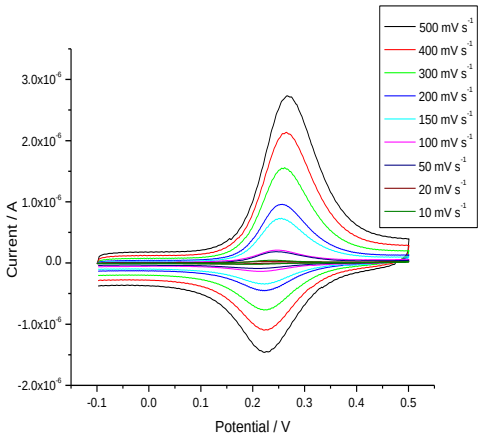
The discrimination between mono nucleotide ferrocene and oligonucleotide ferrocene was originally attributed to differences in the diffusion coefficient of the ferrocene labelled mono-nucleotide compared with the labelled oligo'. However, we now think that this explanation may be false, and more a more subtle explanation accounts for the observed behaviour.

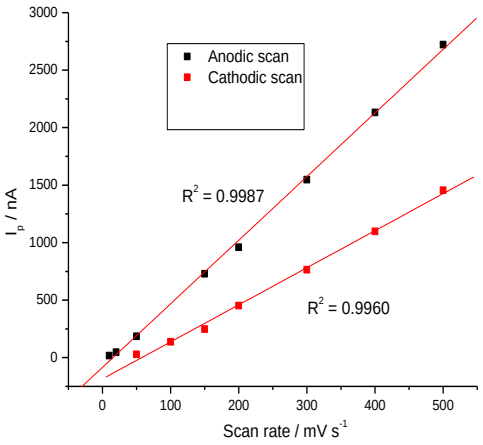
5.4: Cyclic Voltammetric Analysis

Cyclic voltammetry was employed as an electroanalytical tool to probe the processes occurring at the electrode. Screen printed carbon electrodes (SCPEs) from Gwent Electronic Materials Ltd. (GEM) (Pontypool, UK) were employed for electrochemical analysis. The GEM electrodes comprise of screen-printed carbon working and counter electrodes and a silver chloride paste reference electrode upon a valox® backing plastic.

The CTR gene JAOL4 probe and its complementary target were incubated with T7 exonuclease, as per "Methods" and cyclic voltammetry of the digest product yielded

the results illustrated in figure 22a and 22b. The linear relationship between peak current and scan rate is highly indicative of an adsorption controlled electron transfer mechanism and not a diffusion controlled scenario, as initially believed[124].





22a 22b

Figure 22: Evidence of an adsorption-controlled electron transfer mechanism.

Figure 23 displays the results from a study of the non-digested Labelled CTR JAOL4probe, indicating linear dependence of peak oxidation current on the cyclic voltmmetry scan rate, as per figure 22. In this case, however, the peak oxidation currents are a factor of five lower for the same scan rate, suggesting restricted electron transfer from the adsorbed electrochemically Labelled probe to the electrode. Cyclic voltammograms for both the digested and undigested probes show peak separations of greater than the 0 mV expected for surface adsorbed redox moieties, providing further evidence of sluggish adsorption-controlled electron transfer kinetics.

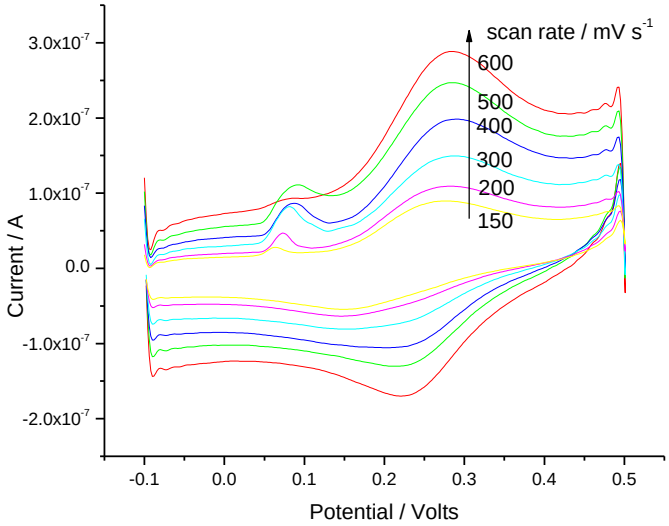
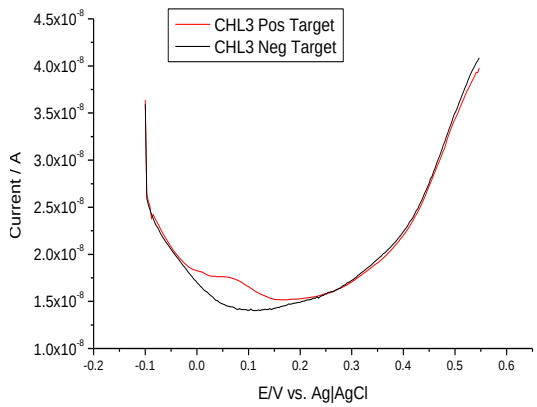


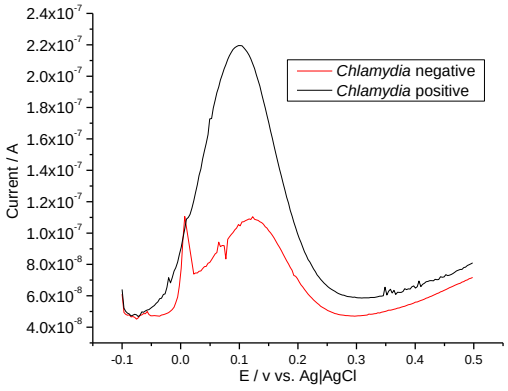
Figure 23: Cyclic voltammetric analysis

Taking into account that the electroactive centres of the labels on the mononucleotide digest fragment and the oligonucleotide probe were both adsorbing to the electrode, it is clear that differences in diffusion will not account for the observed difference in peak oxidation current.

If the response from a glassy carbon electrode and a SPCE is compared, it is clear that a clearer discrimination is observed from a SPCE (Figure 24).



GC electrode



SPCE electrode

Figure 24: Comparison of response on varying electrode surfaces

5.5: Scanning Electron Microscopy of Electrode Surface

The SPCEs were studied using Scanning Electron Microscopy (Figure 25).

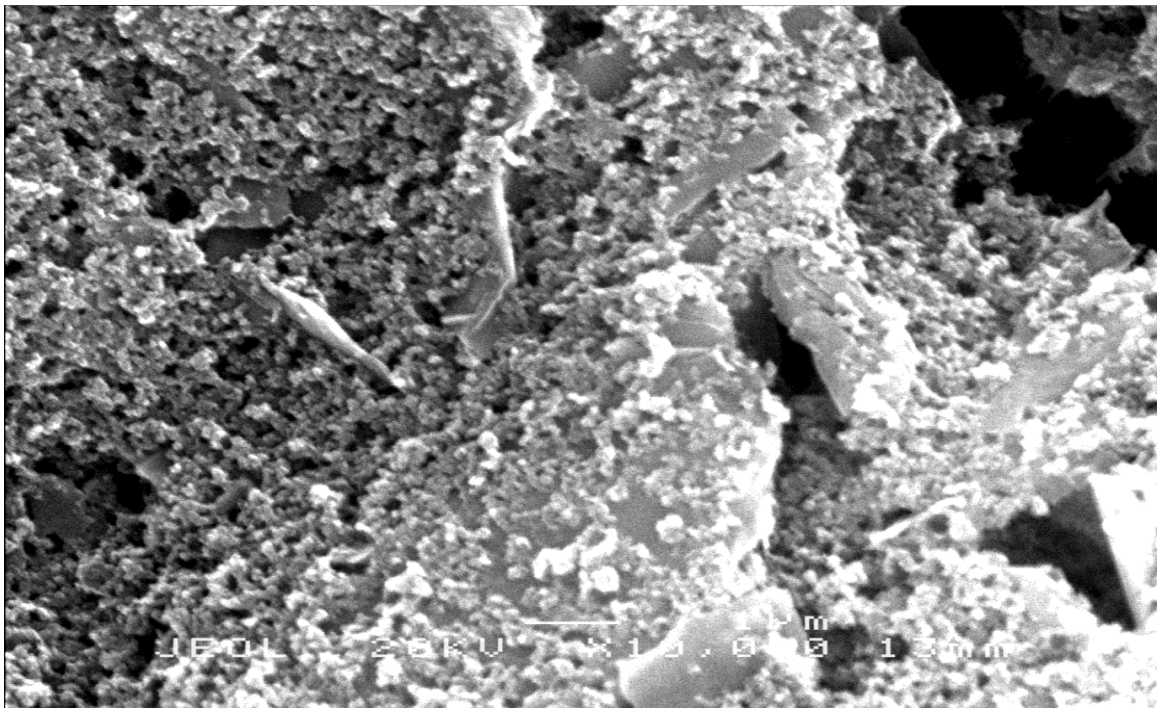


Figure 25: SEM Image of SCPE surface

Figure 25 clearly illustrates the porous open structure of the electrodes, made up of carbon graphite fragments held in a matrix with a PVC binder. The porous nature inherent in the electrode manufacture process not only accounts for the order of magnitude higher currents being measured relative to a planar carbon electrode[125] but may also offer explanation of the discrimination between a labeled mononucleotide and a labeled oligonucleotide.

If the situation that all labeled and unlabelled oligonucleotides and digest fragments are adsorbing to the electrode is considered, in a successful T7 exonuclease digest assay, all fragments will be mononucleotide, or at least smaller fragments of oligonucleotide, depending on the efficacy of the enzyme in the assay conditions. In this case, the labeled mononucleotide has easy access into the porous structure of the electrode matrix, resulting in efficient electron transfer, higher concentrations of electroactive label at the electrode surface matrix and thus higher observed oxidation currents.

In a mis-match assay where no exonuclease activity occurs, the molecular probe will be in tact and strands of oligonucleotide of tens of bases will adsorb onto the electrode surface. If the steric bulk of DNA on the electrode surface in this case is considered along with the anionic repulsion of the DNA layer to oligonucleotide fragments, labeled or unlabelled, access of the electroactive centre of the probe i.e. the label, to the electrode matrix is hindered severely, resulting in poor electron transfer and lower resultant oxidation current.

5.6: E_{ox} – Consideration of Oxidation Potential

Figure 26 illustrates the differential pulse voltammogram of probe CHLJAOL3 in both a positive and negative mungbean endonuclease assay ("Methods").

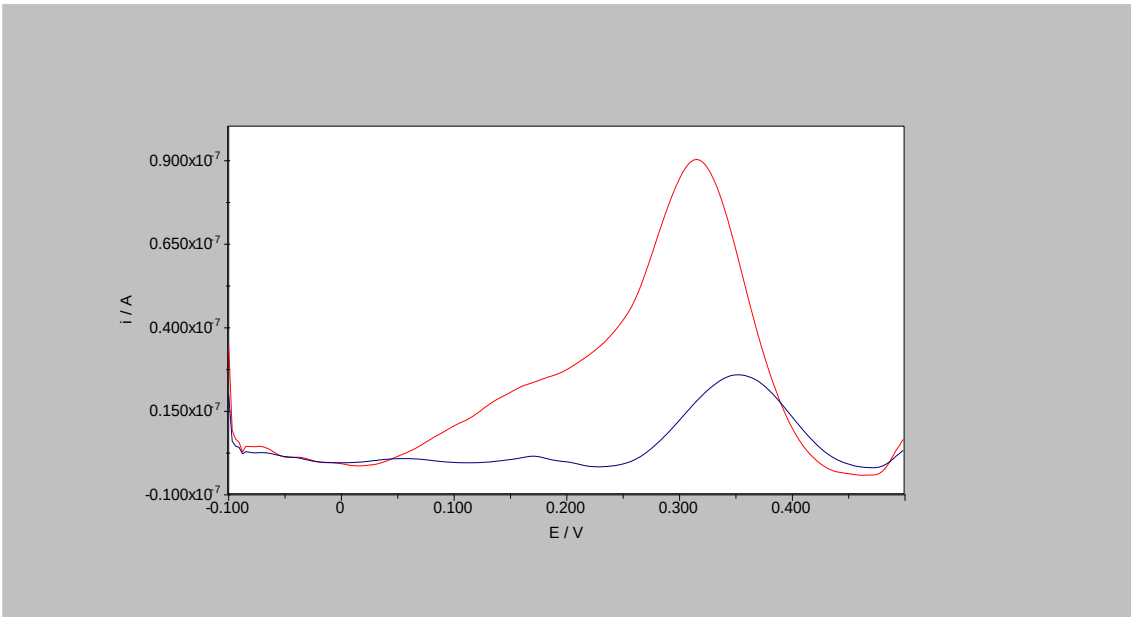


Figure 26: Difference in oxidation potential between digested and undigested fragments

It is interesting to note the difference in potential of the oxidation peaks for the digested and undigested probe of c.25mV. This result also supports the theory of an adsorption-controlled electron transfer mechanism through the porous matrix of the electrode. If we consider the electron transfer from the digested fragment, i.e. labelled mononucleotide, the fragment has easy access to the pores of the electrode and hence less impeded electron transfer to the surface. If we consider the undigested labeled probe however, the oligonucleotide portion of the probe may adsorb onto the

electrode and the electrochemical label, through steric consideration may be relatively far from the electrode itself, as illustrated in Figure 27.

Figure 27 (below) indicates, schematically, the theory of discrimination mechanism.

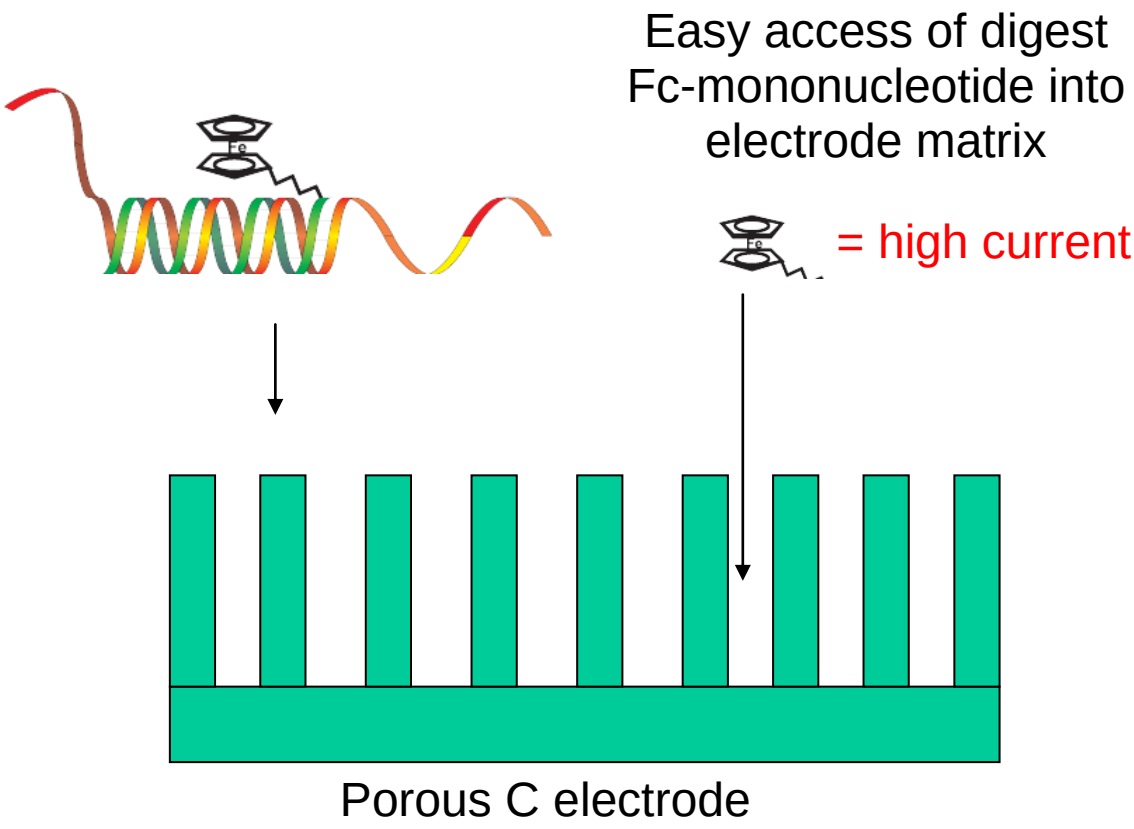


Figure 27: Schematic of electrode process

The electroactive centre of the probe, being relatively far from the electrode surface is unlikely to yield an electron to the electrode directly, but the electron may 'hop' via the DNA electrolytic chain to the electrode surface, resulting in a less efficient transfer mechanism and thus a higher oxidation potential is required in this case. If we also consider the SPCE to have a matricised surface containing pores of various sizes, the effective surface area for electron transfer is also increased. With the digest matrix containing enzyme, proteins and DNA, it is likely that a large degree of

adsorption will occur at the electrode. The model described here renders the electrode accessible to small electroactive moieties through the porous structure and an enhanced electron transfer may occur. Electron transfer through DNA has been thoroughly investigated and has such been widely exploited as a polyelectrolyte in sandwich assays[126].

Chapter 6 - Synthetic Targets

With a range of labels available for potential use in an electrochemical gene detection assay, it was decided that the following labels would be employed:

Probe Name	Label Employed	E_{ox} vs. Ag|AgCl	Target Gene Detected
CHL1	JAOL3	0.075V	Cryptic plasmid of *C. trachomatis*
NG2	JAOL4	0.2V	Opacity gene of *N. gonorrhoeae*
BA3	JAOL1	0.315V	Human beta-actin

As discussed in "Label Design and Synthesis", the label used for probe NG2, consists of a di-ferrocenyl electroactive centre, exhibiting an oxidation peak height of, on experimental average, 6 times that of the monoferrocenyl electrochemical labels developed. In order for the sensitivity of the other probes' oxidation current within the assay not to be compromised by the large current potentially exhibited by label 4, it was decided that the probe was to be used at a 1:10 dilution in H_2O when used in conjunction with other electrochemical probes, as the observed peak currents pre- and post- enzymatic digest were within the same order of magnitude as those exhibited by CHL1 and BA3.

In order to assess the potential applicability of each label to electrochemical gene detection, whether used singularly, or within a multiplex system, dose – response profiles were experimentally established and limits of detection (L.O.D.s) derived.

Synthetic target gene sequences were purchased from Sigma Genosys and pre-diluted in H_2O for use within the following nuclease digest matrix at varying concentrations.

Material	Volume (μL)
H_2O	67
0.1% Tween-20 (in H_2O) (Purchased from Sigma-Aldrich)	10
GE 10xPCR Buffer	10
Probe	3
Synthetic Target	5
T7 Exonuclease	5

The assay was subsequently performed as per "Methods" and the resultant dose – response profiles are displayed (Figure 28).

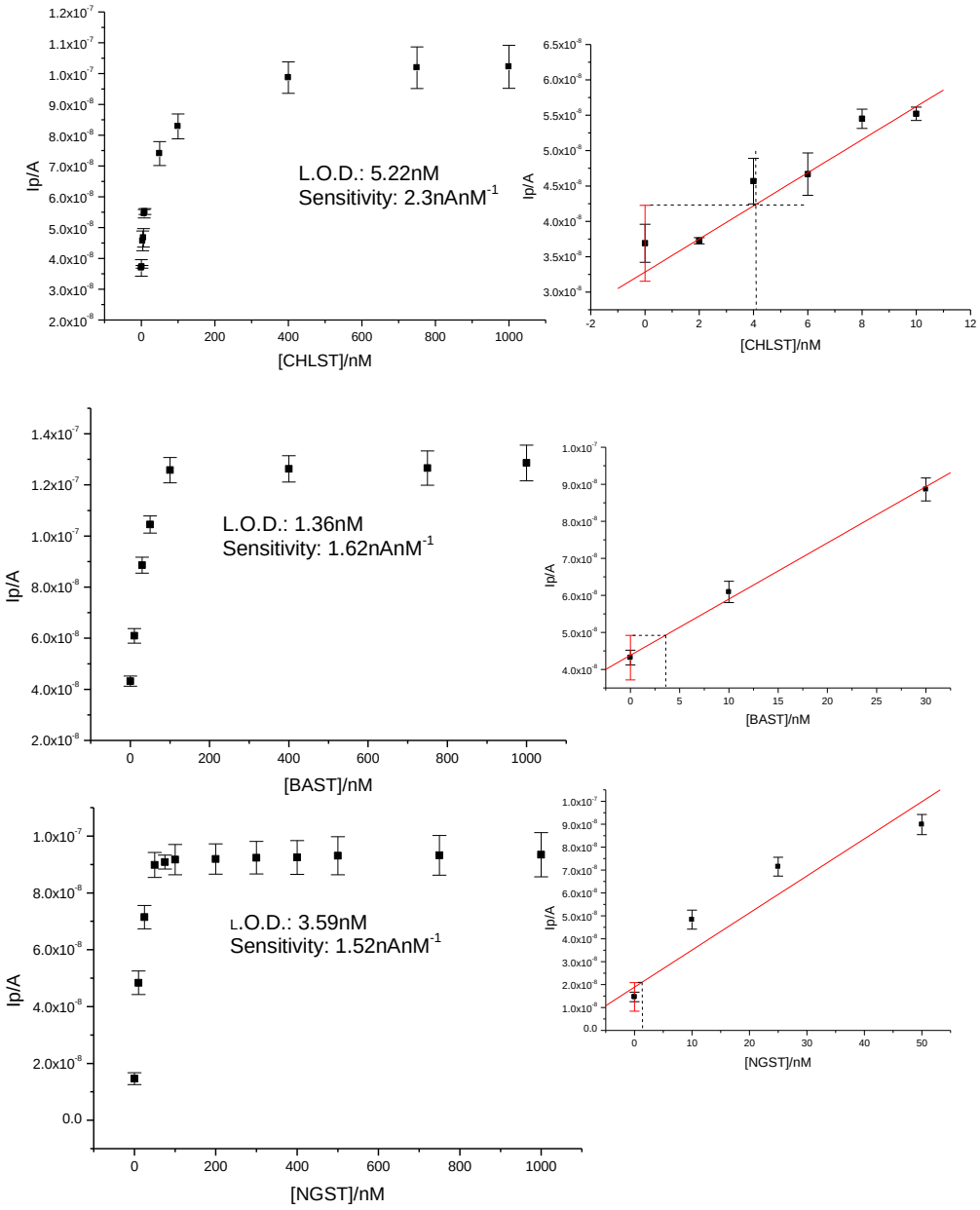


Figure 28: Dose/Response analyses

The sensitivity of the detection assay, experimentally determined here, is somewhat academic. If the application of an assay such as this is considered, the amount of pathogenic DNA presenting a sample becomes unimportant. Furthermore, the amount of pathogenic DNA present will be highly dependent on the sample collection technique and from where, anatomically, the sample is taken. The crucial factor determined in this study is the L.O.D. as it is the absolute presence of the pathogenic DNA that needs to be detected in order for effective treatment to be administered.

L.O.D.s were calculated using the upper value of 3 standard deviations from the mean value for the peak oxidation current obtained when no synthetic DNA target is present in the assay. This value is crucial in terms of assay development as this is deemed the threshold concentration of DNA that PCR must generate in order for the enzymatic electrochemical detection assay to be valid. The values obtained are highly encouraging as these concentrations of DNA are easily obtained during a 40-cycle PCR, as employed in this assay (Methods).

With the validity of each of the electrochemical probes established with the T7 exonuclease detection assay, a triplex synthetic assay, comprising of all three molecular probes was performed as per "Methods". For a negative permutation of the assay, i.e. gene sequence absent, each of the probes' complementary target gene sequence was substituted for a synthetic recognition sequence from the *CTR* gene of *N. meningitidis*. The results of this study are displayed below (Figure 29).

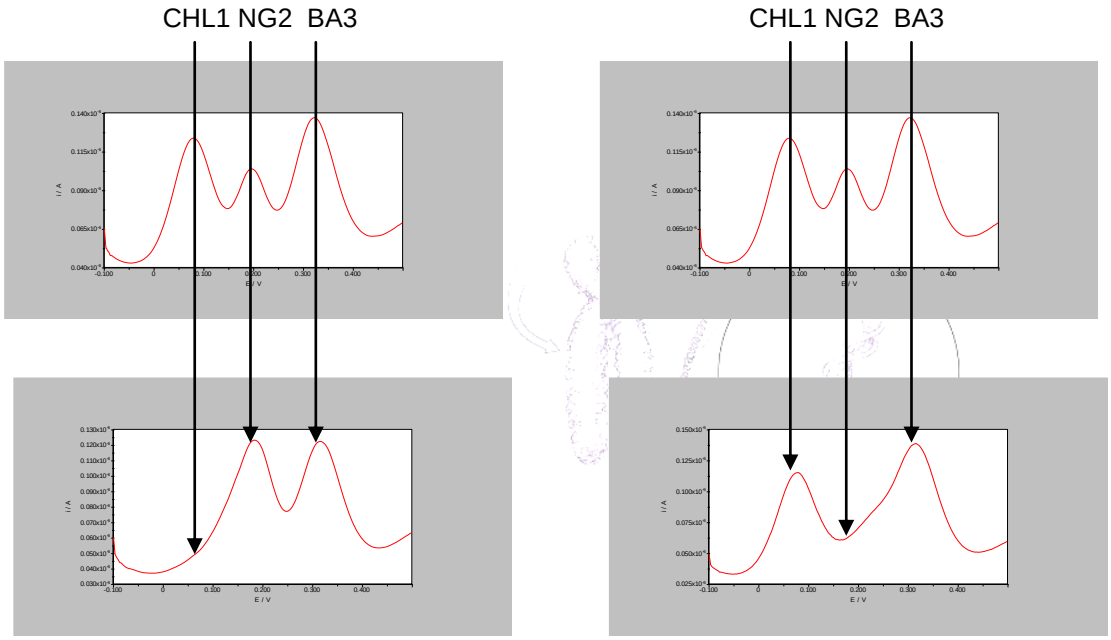


Figure 29: Synthetic Target responses for positive and negative assays

Visually, good discrimination between a gene sequence present (positive) and gene sequence absent (negative) is observed. Statistical analysis supports assay validity from an analytical perspective, with data being valid for positive and negative samples within 3 standard deviations of the mean value obtained for each data set

(five replicate experiments) and a 99.7% confidence level for a true positive or negative result being established. Thus, diagnostic cut-offs do not overlap and the probability of obtaining a false positive or a false negative result is 0.3%.

Chapter 7 - Assay Development using Genomic Targets, Leading to Clinical Screening of Anonymous Patient Samples

7.1 – Initial Analysis of Triplex PCR for Genomic Targets of *C. trachomatis* (CT), *N. gonorrhoeae (NG) and* Human β-actin

Thus far, the assay has been successfully applied to commercially synthesised target fragments for the human β-actin gene, an opacity gene in *Neiserria gonorrhoeae* and the cryptic plasmid in *Chlamydia trachomatis*. In this chapter, we investigate the development of the assay in order to amplify and subsequently detect the above gene sequences using "real", or biological, genomic targets in order to determine whether the assay is suitable for patient sample analyses. Refer to "Methods" for all primer, target and probe sequences referred to herein.

The human β-actin gene was chosen as a target for an internal control for assay validation, owing to the pre-developed PCR conditions and probe-target recognition electrochemistry by Atlas Genetics (formally Molecular Sensing) and the fact that it will be present in any human clinical sample. Refer to "Methods" for details of the sequence and amplicon.

Using the BACTIN3 PCR cycle (Methods), each genomic target was amplified individually in order to assess to effectiveness of the thermal cycling program for each genomic target. The successful conclusion of this test (BACTIN3 could amplify NG and CHL in addition to β-actin) led to the attempt to use this BACTIN3 to amplify two or more target gene sequences at the same time: a duplex or triplex PCR.

An initial triplex PCR was subsequently performed using BACTIN3 cycling parameters, using the amplification mix, detailed in table 9. Negative PCR controls were also run, using all PCR primers, but sigma water was used instead of DNA. In each case, a 20μL reaction volume was used.

	Chlamydia	**Gonorrhoea**	**β-actin**	**Triplex**
Reagent	*Volume (µL)*	*Volume (µL)*	*Volume (µL)*	*Volume (µL)*
10xPCR Buffer (GE – Chalfont St Giles, Buckingham, UK)	2	2	2	2
1.5mMdNTPs (Promega – Southampton, UK)	2	2	2	2
10µM Primer F_{CHL} (Sigma Genosys – Gillingham, Dorset, UK)	1	1	1	1
10µM Primer R_{CHL} (Sigma Genosys)	1	1	1	1
10µM Primer F_{NG} (Sigma Genosys)	1	1	1	1
10µM Primer R_{NG} (Sigma Genosys)	1	1	1	1
10µM Primer $F_{\beta\text{-}A}$ (Sigma Genosys)	1	1	1	1
10µM Primer $R_{\beta\text{-}A}$ (Sigma Genosys)	1	1	1	1
5000UmL^{-1}Taq Polymerase (GE)	0.2	0.2	0.2	0.2
25mM $MgCl_2$ (GE)	1.2	1.2	1.2	1.2
$8.5x10^{-7}$ EBmL^{-1} DNA_{CHL} (US Biological – Swampscott, Massachusetts, USA)	2	0	0	2
10µgmL^{-1} DNA_{NG} (ATCC – Manassas, VA, USA)	0	2	0	2
10nM $DNA_{\beta\text{-}Actin}$ (Sigma Genosys)	0	0	2	2
H_2O (Sigma Aldrich – Gillingham, Dorset, UK)	10.6	10.6	10.6	2.6
Total	20	20	20	20

Table 9: Reagent Volumes for Individual and Triplex PCR of Genomic Targets: Chlamydia trachomatis, Neisseria gonorrhoeae and Human β-actin.

In order to assess whether PCR amplification had been successful, the PCR products were run on an agarose gel, 10µL of the PCR product was added to 2µL of 6x Loading dye (Promega) and the sample was run on a 100mL 3% agarose gel (0.5x TBE), containing 10µL of 10mg/mL ethidium bromide at 100V. Figure 30 illustrates the gel image resultant from this amplification matrix.

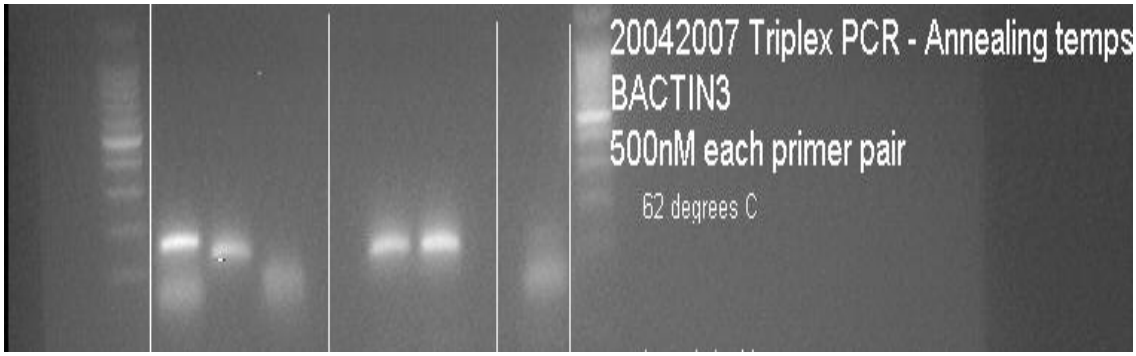


Figure 30: Gel Image of Individual and Triplex PCR of Genomic Targets for Chlamydia trachomatis, Neisseria gonorrhoeae and Human β-actin – Lane 1: DNA Ladder, Lane 2: Chlamydia (156bp), Lane 3: β-actin (120bp), Lane 4: Gonorrhoea (90bp), Lanes 5 & 6: Triplex PCR, Lanes 7: Negative Control & Lane 8: DNA Ladder.

Lanes 2 to 4 exhibit clear bands of PCR product, relating to each genomic target amplified within the PCR matrix. There is, however, residual primer dimerisation product in lanes 2, 4 and 7. This is attributable to the primer pairage being designed for uniplex PCR with no re-optimisation for a triplex PCR. The triplex PCR, bands, however, (Lanes 5 and 6), showed preferential amplification of β-actin, with no apparent amplification of the other PCR targets. This problem of non-amplification of the CHL and NG targets was subsequently investigated.

7.2 – Development of Triplex PCR for *Chlamydia trachomatis, Neisseria gonorrhoeae and Human* β-actin

Gel Imaging of PCR in PCR Optimisation Study

There are many factors to consider when designing a successful multiplex PCR, including magnesium concentration, primer concentration and primer annealing temperature. In order to assess the comparative annealing temperatures of the primers used in this triplex PCR, bioinformatic software from IIT Biotech (Germany) was used[120]. Table 10 displays the annealing temperature of each primer used in the triplex PCR.

Primer	Sequence (5'-3')	Annealing Temperature T_m
Chlamydia Forward Primer (CHLF)	CCCAAGCAACATCTCGGTACA	61°C
Chlamydia Reverse Primer (CHLR)	TGAAGCTGTAGCCGCGCTCGGT	59.1°C
Beta-actin Forward Primer (BAF)	TTGAAGCTGTAGCCGCGCTCGGTAG	69.58°C
Beta-actin Reverse Primer (BAR)	TTCACCCACACTGTGCCCATCTACGAAG	68.0°C
Gonorrhoea Forward Primer (NGF)	GTTGAAACACCGCCCGG	62.0°C
Gonorrhoea Reverse Primer (NGR)	CGGTTTGACCGGTTAAAAAAAGAT	57.4°C

Table 10: Annealing Temperatures of Primers Used in Triplex PCR.

The annealing temperature in the thermal cycling program used (BACTIN3) was set at 60°C. It was therefore important to ensure that in order for each PCR target to become successfully amplified, each set of annealing temperatures for each primer pair as well as each genomic target is as similar as possible.

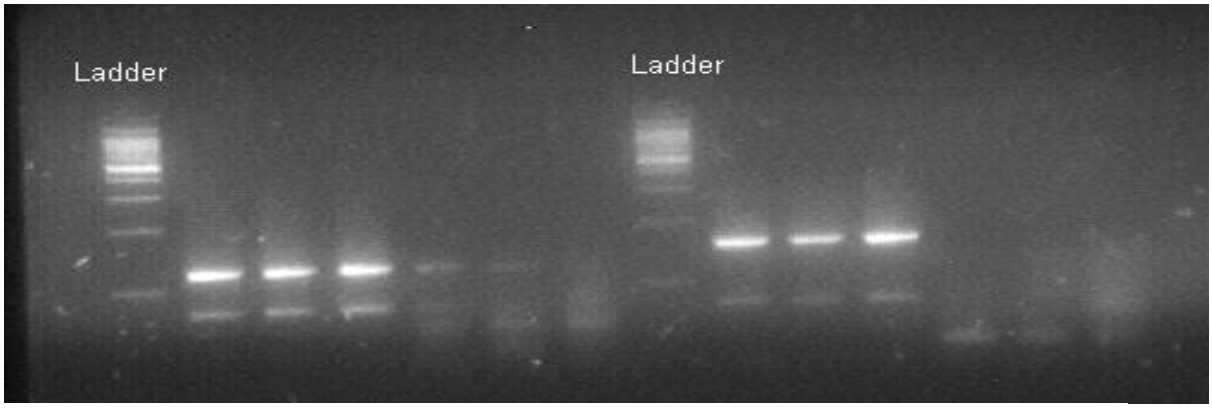
If we consider the NGR, the annealing temperature is below that of the one set for the thermal cycling program and as such, amplification of this gene sequence is hindered, as observed in gel image BX. Note also the relatively high annealing temperatures of the primers for the β-actin gene sequence. Higher annealing temperatures will encourage successful hybridisation of these primers relative to the other targets and thus competitive amplification occurs, in this case, in favour of the β-actin target amplification (Figure 30).

In order to address this imbalance, the IIT Biotech bioinformatic software[127] was employed to re-design the anomalous primers, in conjunction with the gene target sequences. Table 11 illustrates the resultant changes.

Superseding Primer	Superceded Primer	Sequence (5'-3')	Annealing Temperature T_m
BAF2	BAF	GAAGCTGTAGCCGCGCTCG	64.0°C
BAR2	BAR	CACCCACACTGTGCCCATCTAC	64.5°C
NGR2	NGR	TCCTTATTCGGTTTGACCGGTTAAAA	60.0°C

Table 11: Primer Modification with Resultant T_m.

In order to further elucidate factors affecting the near simultaneous co-amplification of all genomic targets in the triplex PCR, duplex PCRs for *N. gonorrhoeae* with β-actin and *N. gonorrhoeae* with *C. trachomatis* were performed in 30µL reaction volumes, using 300nM of NGF and NGR2 primers and 300nM of the other primers (i.e. all primers were used in equimolar amounts). PCR product (10µL) was added to 2µL of 6x loading dye (Promega) and the sample was run on a 100mL 3% agarose gel (0.5x TBE), containing 10µL of 10mg/mL ethidium bromide at 100V. Figure 31 illustrates the gel image resultant from this amplification matrix. It can be seen in figure 31 that both NG and BA and NG and CT could be amplified simultaneously using the optimised primers and the BACTIN3 thermal cycling program.



Gonorrhoeae + Beta-Actin *Gonorrhoeae + Chlamydia*

AD 030407 Two duplex tests with Gonorrhoeae

Figure 31: Duplex PCR Gel Electrophoresis – Lane 1: DNA Ladder, Lanes 2-4: N.gonorrhoeae (84bp) with beta-actin (118bp), Lanes 5-7: Negative Control, Lane 8: DNA Ladder, Lanes 9-11: N.gonorrhoeae (84bp) with Chlamydia (156bp), Lanes 12-14 Negative controls

Electrochemical Determination of PCR products from PCR Optimisation Study

To the remaining 20µL of the PCR product, 1µL of each probe [CHL3 (75mV), Ng14-151(1:10) (200mV) and BA1(315mV)] was added, followed by 2µL of T7 exonuclease and the matrix incubated as per Inc-J program (37^{o}C for 20 minutes, followed by cooling to 16^{o}C). Electrochemical measurement was then performed using the Autolab with electrochemical parameters as detailed in "Methods". Resultant voltammograms and gel image are displayed in Figure 32.

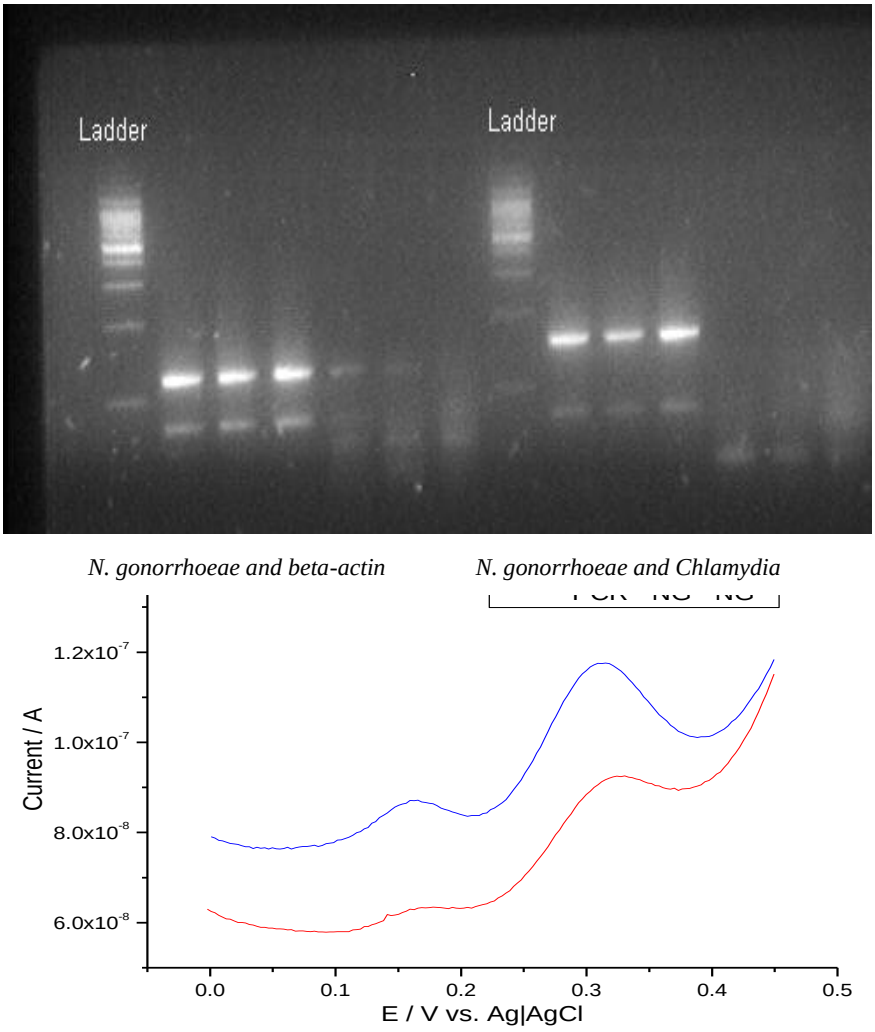


Figure 32: Gel Image and Voltammograms showing differences in duplex compatibility with respect to NG. Both traces show the NG peak at 170mV – voltammogram measured in the presence of probes for Gonorrhoea and Beta-Actin. Blue trace is a voltammogram resulting from co-amplication of N. gonorrhoeae and beta-actin and the red trace results from co-amplication of N. gonorrhoeae and Chlamydia.

Figure 31 illustrates that the amplification of *N. gonorrhoeae* is amplified to a greater extent when mixed with β-actin than with *Chlamydia*, as indicated by the brightness of the band (84bp) as well as the enhanced peak for *N. gonorrhoeae* (170mV) following T7 enzymatic digestion (Figure 32). As a consequence, the concentration of the primer pairage for *Chlamydia* was reduced to 100 nM, and a triplex PCR was performed using the modified primers and the reduced concentration of Chlamydia primers, as detailed in table 12.

	Triplex
Reagent	*Volume (µL)*
10xPCR Buffer (GE – Chalfont St Giles, Buckingham, UK)	3
1.5mMdNTPs (Promega – Southampton, UK)	3
10µM Primer F_{CHL} (Sigma Genosys – Gillingham, Dorset, UK)	0.3
10µM Primer R_{CHL} (Sigma Genosys)	0.3
10µM Primer F_{NG} (Sigma Genosys)	0.9
10µM Primer R_{NG} (Sigma Genosys)	0.9
10µM Primer $F_{\beta\text{-}A}$ (Sigma Genosys)	0.9
10µM Primer $R_{\beta\text{-}A}$ (Sigma Genosys)	0.9
5000UmL^{-1}Taq Polymerase (GE)	0.3
25mM $MgCl_2$ (GE)	1.8
$8.5x10^{-7}$ EBmL^{-1} DNA_{CHL} (US Biological – Swampscott, Massachusetts, USA)	2
10µgmL^{-1} DNA_{NG} (ATCC – Manassas, VA, USA)	2
10nM $DNA_{\beta\text{-}Actin}$ (Sigma Genosys)	2
H_2O (Sigma Aldrich – Gillingham, Dorset, UK)	11.7
Total	30

Table 12: Reagent Volumes for Triplex PCR of Genomic Targets: Chlamydia trachomatis, Neisseria gonorrhoeae and Human β-actin.

The PCR amplification was performed on a PCR Block, using the BACTIN3 program. 10µL of the PCR product was added to 2µL of 6x Loading dye (Promega) and the sample was run on a 100mL 3% agarose gel (0.5x TBE), containing 10µL of 10mg/mL ethidium bromide at 100V.

Figure 33 illustrates the gel image corresponding to this experiment. As evidence of preferential amplification of beta-actin in this case remained, a further triplex PCR

was performed, as per Table 12, but reducing the β-actin primer pairage to 100nM. This is shown on the right hand side, lanes 8-10. As observed in the gel image, an even amplification of all three genomic targets resulted, with no evidence of competitive amplification.

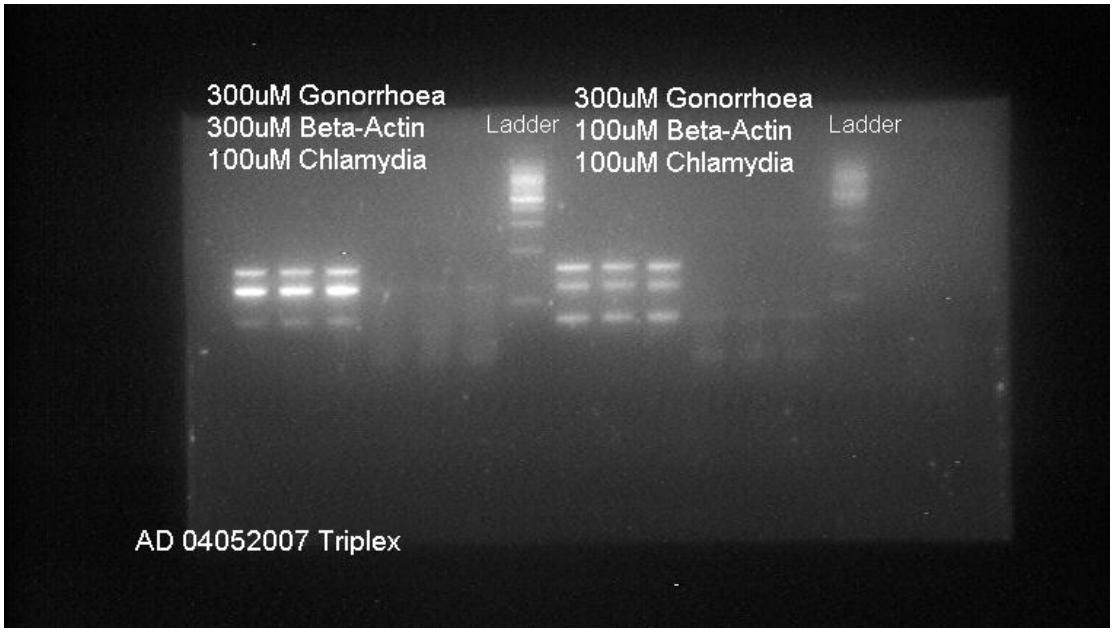


Figure 33: Gel Image of Triplex PCR of Genomic Targets for Chlamydia trachomatis(156bp), Neisseria gonorrhoeae(84bp) and Human β-actin(118bp) – Lanes 1-3: Triplex PCR, Lanes 4-6: Negative Control, Lane 7: DNA Ladder, Lanes 8-10: Triplex PCR, Lanes 11-13: Negative Control, Lane 14: DNA Ladder.

7.3 – Evaluation of the Electrochemical Triplex Assay

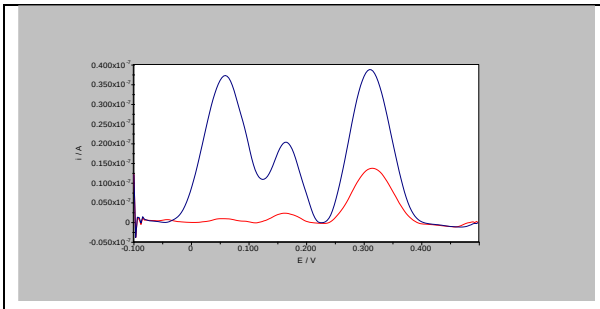
With an established successful methodology for amplifying the three target gene sequences together in a triplex PCR, electrochemical analyses of the samples, along with statistical analysis becomes a crucial stage in the assay development. Confidence levels for the genomic analyses become important when dealing with patient samples as reference ranges need to be established in any clinical diagnostic protocol. In the "Synthetic Targets" chapter, the confidence level for an electrochemical result using synthetic gene targets was calculated to be 99.7%, based upon a Gaussian distribution.

With the added complexity involved in a biological assay involving multiplex amplification, the assay required re-evaluation in terms of the confidence levels

associated with an electrochemical result. To this end, ten triplex PCRs were performed – 5 positive and 5 negative, as described in the methodology outlined in section 4.2. Negative controls were performed using Sigma water in lieu of genomic DNA and 10µL of the PCR product was added to 2µL of 6x Loading dye (Promega) and the sample was run on a 100mL 3% agarose gel (0.5x TBE), containing 10µL of 10mg/mL ethidium bromide at 100V in order to confirm successful amplification of the positive samples and non-contamination of the negative samples.

To the 20µL of the PCR product, 1µL of each probe [CHL3 (75mV), Ng14-151(1:10) (200mV) and BA1(315mV)] was added, followed by 2µL of T7 exonuclease and the matrix incubated as per Inc-J program (37^{o}C for 20 minutes, followed by cooling to 16^{o}C for ever). Electrochemical measurement was then performed using the autolab with electrochemical parameters as detailed in "Methods".

Figure 34 illustrates a voltammogram of a negative triplex PCR (red trace), overlaying a positive triplex PCR (blue trace) (34A), with the analogous gel image (34B)



34A: Triplex Electrochemical Voltammogram – Blue Trace – Positive Triplex Assay, Red Trace – Negative Triplex Assay.

34B: Triplex Gel Image – Lane 1: DNA Ladder, Lanes 2-4: Positive Triplex, Lanes 5-6: Negative Control, Lane 7: DNA Ladder.

Experimentally, confidence in a result using this particular genomic assay is limited to one standard deviation, as at two standard deviations from the mean, the diagnostic cut-offs overlap. Thus, a 68% confidence level in the assay is defined, based on the Empirical rule of Gaussian distribution. Note how this confidence level is much

lower than that calculated for the synthetic target assay (99.7%), owing to the greater complexity of the biological case.

Further work would be well spent investigating factors that could improve the assay confidence levels when working with genomic targets.

7.4 – Analysis of Clinical Samples from the Royal Free Hospital, London

The final part of this work looked at whether the assay could be used to identify presence or absence of bacteria in clinical samples

Clinical samples were obtained from Dr. Clare Ling, Dept. Virology at the Royal Free Hospital in London, UK. The samples were anonymous discard samples, which had been tested using a culture assay for *Neisseria gonorrhoeae* and using the BD Probetec™ protocol for *Chlamydia trachomatis*. The *BD ProbeTec Chlamydia trachomatis* amplified DNA assay is based on the simultaneous amplification and detection of target DNA using amplification primers and a fluorescently labelled detector probe. The amplification reagents are dried in two separate disposable microwell strips. The processed sample is added to the Priming Microwell which contains the amplification primers, fluorescently labelled detector probe, and other reagents necessary for amplification. After incubation, the reaction mixture is transferred to the Amplification Microwell, which contains two enzymes (a DNA polymerase and a restriction endonuclease) necessary for Strand Displacement Amplification (SDA) a particular amplification technique used widely in commercial DNA amplification-based diagnostics. The Amplification Microwells are sealed to prevent contamination and then incubated in a thermally controlled fluorescent reader which monitors each reaction for the generation of amplified products. The presence or absence of CT is determined by relating the *BD ProbeTec* MOTA (Method Other Than Acceleration) scores for the sample to pre-determined cutoff values. The MOTA score is a metric used to assess the magnitude of signal generated as a result of the reaction.

The patient samples provided were 8 tested negatives for *C. trachomatis* and 6 tested positives for *C. trachomatis*. All patients, however, were confirmed as negative for *N.*

gonorrhoeae. Using the *BD ProbeTec* protocol, patient samples, whether they are urine or in a swab elution buffer, were initially centrifuged for thirty minutes at 2000 g and the supernatant carefully decanted in order to minimize any inhibition of the amplification stage. The samples were then lysed at 114^{o}C. Of each patient, two sub-samples were provided, one lysed and one unlysed. The lysed samples in each case, were used in the assay in order to improve amplification.

Table 13 indicates patient numbers for the samples provided, including the result of their analysis at the Royal Free Hospital. It should be noted that the assay was performed 'blind'. It was not until later discussion with the RFH that the veracity of our assay was established.

Samples Typed Negative for *C. trachomatis*	07M195165 07M195167 07M195175 07M195172 07M195180 07M195179 07M195178 07M195176
Samples Typed Positive for *C. trachomatis*	07M194999 07M195129 07M195831 07M195139 07M195123 07M195149

Table 13: Sample Identification

7.4.1 – Detection of *C. trachomatis* in a clinical sample

Initially, it was important to establish whether Chlamydia could be detected in a clinical sample. To this end, samples 07M195123 (Positive) and 07M195167 (Negative) were subjected to PCR amplification, using the BACTIN3 thermal cycling program and the reaction matrix displayed in Figure 35. PCR negative controls were also performed using sigma water.

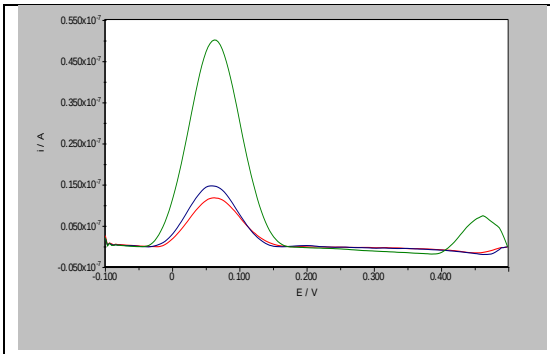
	Triplex
Reagent	*Volume (µL)*
10xPCR Buffer (GE – Chalfont St Giles, Buckingham, UK)	3
1.5mMdNTPs (Promega – Southampton, UK)	3
10µM Primer F_{CHL} (Sigma Genosys – Gillingham, Dorset, UK)	0.3
10µM Primer R_{CHL} (Sigma Genosys)	0.3
5000UmL^{-1}Taq Polymerase (GE)	0.3
25mM $MgCl_2$ (GE)	1.8
BD Probec Sample (Royal Free Hospital – London, UK)	2
H_2O (Sigma Aldrich – Gillingham, Dorset, UK)	19.3
Total	30

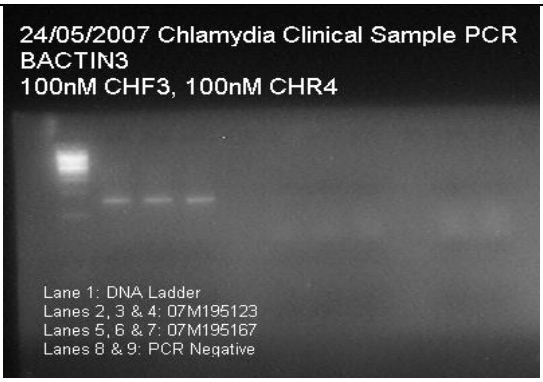
Figure 35: PCR Components for Clinical Sample PCR

10µL of the PCR product was added to 2µL of 6x Loading dye (Promega) and the sample was run on a 100mL 3% agarose gel (0.5x TBE), containing 10µL of 10mg/mL ethidium bromide at 100V.

To the remaining 20µL of the PCR product, 1µL of each probe (CHL3, Ng14-151(1:10) and BA1) was added, followed by 2µL of T7 exonuclease and the matrix incubated as per Inc-J program (37^{o}C for 20 minutes, followed by cooling to 16^{o}C). Electrochemical measurement was then performed using the Autolab with established electrochemical parameters (Methodologies).

Figure 36 displays a voltammogram of the PCR Negative (red trace), overlaying a negative amplified patient sample (07M195167 - blue trace) and the positive amplified sample (07M195123 - green trace) (36A), with the analogous gel image (36B).





36A: Chlamydia Voltammogram

36B: Chlamydia PCR Gel Image

7.5 Chlamydia with beta-actin (Internal Control) gene

To samples 07M195167 (patient negative for CT) and 07M195123 (patient positive for CT), and a duplex amplification of chlamydia with β-actin was performed, using the parameters as decribed in "Methods". β-actin was spiked into the PCR mixture (as detailed per "Methods"), at a concentration of 10nM prior to addition into the PCR reaction.

Figure 37 displays a voltammogram of the PCR Negative (red trace), overlaying a negative amplified patient sample (07M195167 - blue trace) and the positive amplified sample (07M195123 - green trace) (inset into figure), with the analogous gel image.

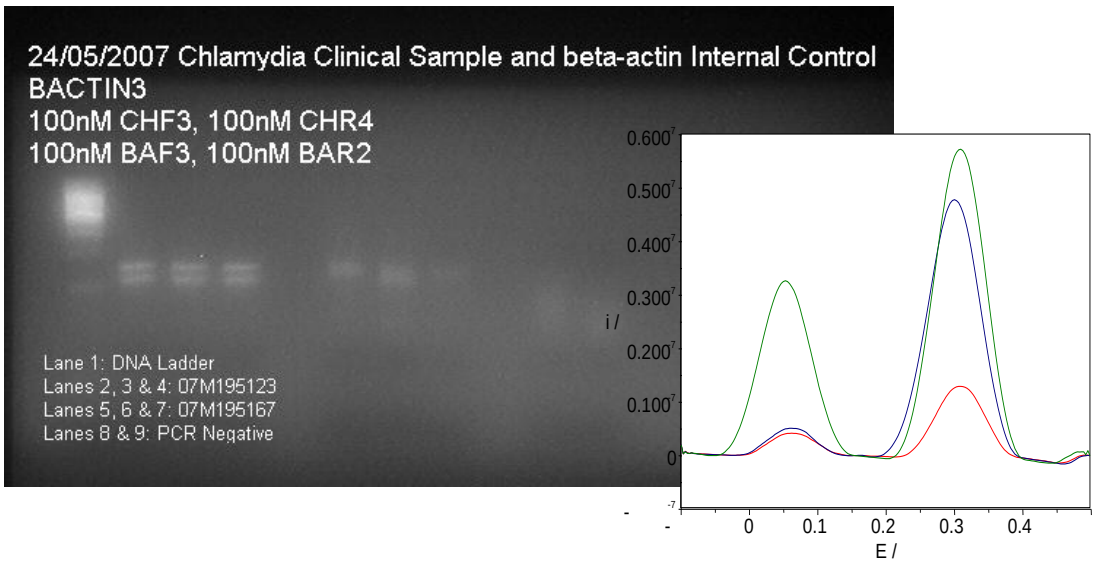


Figure 37: Gel Image and Voltammogram from Duplex PCR of Clinical Samples

7.6 Screening of clinical samples for CT and NG

Each clinical sample was subsequently tested for the presence of chlamydia and gonorrhoea, using a triplex amplification as detailed in "Methods". The results of the study are illustrated in Figure 38.

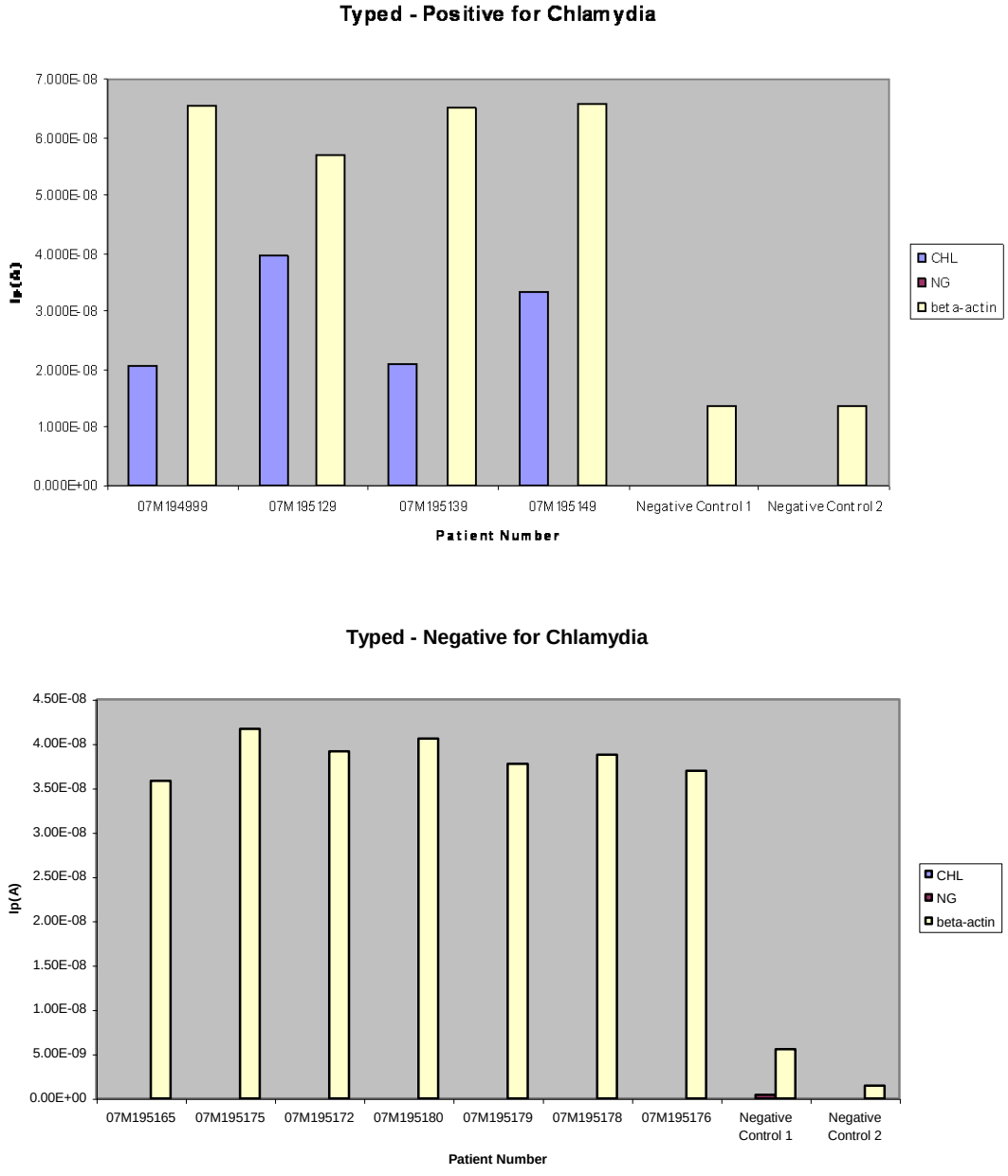


Figure 38: Results from the Clinical Samples Screen for NG and CT with β-actin internal control gene.

The amplification of each sample was confirmed by agarose gel electrophoresis and visualised by UV light. As observed, successful amplification and detection of both

chlamydia and β-actin occurred in the chlamydia-postive samples, whilst only β-actin was detected in the chlamydia-negative samples.

The absence of gonorrhoeal infection for the negative samples was subsequently confirmed by the Royal Free Hospital, hence indicating the veracity of the assay in this case.

We have thus, from clinically-gained patient samples, successfully amplified chlamydial inclusions and an introduced internal control gene. Furthermore, using the T7 exonuclease electrochemical digest assay, we have managed to discriminate, with confidence, patients validated as either positive for chlamydial infection or negative, as validated by commercially-used clinical diagnostics.

Further investigation would be well spent using typed positive gonorrhoeal and chlamydial samples in order to strengthen the validity of the assay. The use of human beta-actin inherent in the collected clinical samples, instead of artificial addition as an internal control for the assay would also be beneficial and further work could substantiate this.

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